DEVELOPMENT OF A NONCURED PORK PRODUCT WITH THE POTENTIAL FOR NONREFRIGERATED STORAGE

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1997

In loving memory of Durward W. Woolsey, Sr., my grandfather.

ACKNOWLEDGEMENTS

Glory, honor and praise belong to my Lord and Saviour, Jesus Christ. I have taken refuge in Him; He has never let me be put to shame; He has delivered me into His righteousness. He has not handed me over to the enemy but has put my feet in a spacious place (Psalm 31:1&8, NIV). He has sustained me through some difficult spiritual and emotional trials. He has used these trials to develop perseverance, character and hope within me (Romans 5:3&4, NIV).

Appreciation is extended to Dr. R.L. West, chairman of the supervisory committee, for the guidance, wisdom and patience he has shown me during the duration of my program. Thanks are also extended to Dr. D.D. Johnson, Dr. F.W. Leak, Dr. S.K. Williams, Dr. J.A. Lindsay and Dr. S.F. O'Keefe for their efforts and guidance as a supervisory committee. Special thanks are extended to Dr. West, Dr. Johnson and Dr. Williams for funding this project.

The friendships of Debbie Neubauer, Mary Beth Fort and Larry Eubanks are appreciated and have contributed to my enjoyment of this program. Debbie Neubauer, Anna Zometa, Pam Royer and Doris Sartain are appreciated for their assistance in the laboratory. Larry Eubanks and the Meat Laboratory crew are appreciated for their assistance in livestock slaughter and product preparation.

The friendships of fellow graduate students, Claudio Alvarez, N.T. Cosby, Mike Coker, Will Rhodes, Jeff Arseneau, Maria Rivas, Joanna Harrelson, Brenda Allen and

Victoria D'Costa have added a special yet unexplainable twist to my doctoral program. Special thanks are extended to Maria Rivas, Joanna Harrelson and Brenda Allen for their assistance during football Saturdays.

My parents, Durward and JoAnne Woolsey, are greatly appreciated for instilling qualities of commitment, persistence, honesty, integrity and servanthood into me as a young man. Their moral and occasional financial support during this program have been invaluable to me.

My friends, brothers and sisters in Christ, at InterVarsity Christian Fellowship and The First Assembly of God, Gainesville, are appreciated for the love and encouragement they have given me over the past several years. Special thanks are extended to Eric Koenig, Shad Smith, Laura Farrow and Jim Capen who often simply listened.

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Abstract of Dissertation Presented to the Graduate School of the
University of Florida in Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy

DEVELOPMENT OF A NONCURED PORK PRODUCT WITH THE POTENTIAL FOR NONREFRIGERATED STORAGE

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August 1997

Chairman: R.L. West
Major Department: Animal Science

Combinations of four preservatives were evaluated for their ability to inhibit the growth of selected foodborne pathogens. The most effective preservative combinations were then utilized in noncured, restructured pork rolls to compare their antimicrobial activity with sodium nitrite. The study was divided into four sections: 1) the effects of sodium lactate (SL), glucono-delta-lactone (GDL), disodium ethylenediaminetetraacetate (EDTA), Nisaplin (NN) and their combinations on the growth of *Escherichia coli* O157:H7; 2) the effects of SL, GDL, NN and their combinations on the growth of selected foodborne pathogens; 3) the effects of SL, GDL, EDTA and their combinations on the growth of *E. coli* O157:H7 and *Salmonella typhimurium* and 4) the utilization of SL and GDL in nonrefrigerated and refrigerated, restructured pork rolls.

Independently, NN inhibited the growth of *Listeria monocytogenes* and *Staphylococcus aureus*. However, NN did not inhibit the growth of *S. typhimurium* or

Yersinia enterocolitica. In addition, NN did not exhibit synergistic interactions with EDTA, GDL or SL toward the inhibition of *S. typhimurium*, *Y. enterocolitica* or *E. coli* O157:H7. Antagonistic interactions occurred between NN and combinations of SL and GDL when applied to *E. coli* O157:H7 and *S. aureus*. NN was removed from the study because of its narrow range of activity and antagonistic interactions with GDL and SL.

When utilized at 0.05, 0.372 and 0.745%, EDTA exhibited synergistic interactions with GDL, SL and their combinations toward the inhibition of *E. coli* O157:H7 and *S. typhimurium*. However, EDTA's synergistic interaction with SL and GDL was lost when EDTA levels were reduced to 0.0036 and 0.0072%. EDTA was removed from the study because it is only permitted in cooked sausage products at 0.0036% and was not effective at this level.

Combinations of SL and GDL exhibited synergistic interactions that were inhibitory toward the growth of all of the pathogens investigated. The proposed mechanism for the GDL*SL interaction is an equilibrium between GDL and lactic acid (from SL), that increases lactic acid's ability to penetrate the bacterial cell membrane.

Restructured pork rolls were produced with combinations of SL and GDL and compared to two control groups, one control group contained sodium nitrite while the other did not. Sensory analysis indicated that SL and GDL enhanced pork flavor intensity and saltiness and increased the perception of bitter and acid off-flavors in the pork rolls. After 12 days of nonrefrigerated (18 to 22°C) storage, the control groups exhibited greater than 10^5 CFU/g of aerobic and anaerobic growth while the pork rolls containing SL and GDL exhibited less than 10^2 CFU/g of growth.

CHAPTER 1
INTRODUCTION

Refrigeration (4°C) is one of the most common means of preserving meat; however, in developing countries, refrigeration facilities are often lacking. The environmental conditions in most developing countries favor microbial growth, causing meat to spoil rapidly and shortages in the availability of meat proteins (Guerrero and Taylor, 1994; Ogden et al., 1995). Before the invention of refrigeration, meat was traditionally preserved by reducing its pH and/or water activity (a_w), by drying, fermentation and/or the addition of chemical preservatives such as salt, sugar, nitrates, nitrites and smoke. In developed nations that have wide access to refrigeration, these or similar processes are used to manufacture specialty items. However, in developing countries such processes and their resulting products are a necessity to maintain a regular source of meat protein in the human diet.

The products resulting from these processes typically have a a_w of 0.70-0.90 and are classified as intermediate moisture food, or a a_w of < 0.70 and are classified as dry food. For developing countries, intermediate moisture foods provide advantages of reduced spoilage, improved safety and increased shelf-life without refrigeration or thermal processing. They can also be eaten as is, without rehydration, because they retain a soft moist texture (Erickson, 1982).

Some intermediate moisture meats common in the United States are country cured ham, beef jerky, various fermented sausages (Genoa salami, pepperoni, hard salami, summer sausage, etc.), Proscutti ham and salted fish. Traditional intermediate moisture meats have been developed in tropical and developing countries. *Dendeng* is a dried beef product prepared from sliced round, coconut sugar, salt and spices that is common to Indonesia and some regions of Asia (Purnomo et al., 1983). *Kilishi* is a sun-dried product prepared from beef hindquarter meat, mutton or goat meat and groundnut cake powder and seasoning. It is traditional to Nigeria and is exported to Saudi Arabia (Igene, 1988). *Beef cecina* is a traditional Mexican product. It is salted strips of hindquarter sliced in the direction of the muscle fibers and held under refrigeration for distribution. Depending upon the region in which *cecina* is produced it may be coated with vinegar, safflower oil or lard and undergo a drying process of 4 to 24 hr at ambient temperature or light sun-drying. (Reyes-Cano et al., 1994). In Asia, fish are generally preserved by sun-drying or salting/sun-drying. In Africa, fish are preserved by smoke drying where the fish are subjected to heat (120°C and greater) and smoke from a fire (Ward, 1995).

In attempts to develop new intermediate moisture meat products and increase availability of meat protein to human diets, traditional preservation methods have been applied to nontraditional products. Intermediate moisture meat products utilizing water buffalo (*Bos bubalis*) round, salt and glycerol, glycerol and nitrite, or nitrite were developed in a series of studies (Prabhakar and Ramamurthi, 1990; Prabhakar et al., 1992a and 1992b). The products exhibited pH values of 5.79-6.12, a_w values of 0.85 and a moisture content of 41-46%. The product exhibited a 60-day shelf-life with no evidence of spoilage when stored at ambient temperature (25-40°C). The color of the products containing glycerol had a slight yellow hue while the nitrite product was more red.

Dzudie and Okubanjo (1992) cured rabbit meat (leg, shoulder, loin and rib cuts) using methods similar to those of country cured hams. After smoking and 4 weeks of room temperature (25-29°C) aging, the rabbit meat was 32-36% moisture, 13-18% salt and had a pH of 6.10-6.30. The curing yields were 45 to 60%. Some of the problems with dried and/or salted meat are that they typically have low processing yields, they often require some rehydration, some of the salt needs to be removed from the product to increase palatability and the physical characteristics of the meat are changed from those of fresh meat.

In order to preserve fresh meat, Guerrero and Taylor (1994) proposed applying lactic acid bacteria (*Lactobacillus* and *Pediococcus*) on the exterior of fresh meat cuts. Lactic acid bacteria have been traditionally used to produce fermented sausages. During fermentation, lactic acid bacteria grow and produce lactic acid and antimicrobial agents which inhibit the growth of spoilage and pathogenic bacteria. Guerro et al. (1995) explored the viability of this concept at semitropical conditions (25°C). *Lactobacillus bulgaricus* and *Pediococcus pentosaceous* overgrew *Pseudomonas* spp. when they were inoculated on beef and pork cuts that had been immersed in a 10% sucrose solution and wrapped with a polyvinylchloride film. A commercial starter culture consisting of *Lactobacillus plantarum* and *Micrococcus kristinae-varians* reduced the growth rate of *Pseudomonas* spp. without notably affecting meat color or degree of lipid oxidation.

Meat preserved via drying, smoking, salting and/or fermentation typically exhibits physical and sensory characteristics that are quite different from cooked fresh meat. Therefore, there is the need to develop shelf-stable meat products that have physical and sensory characteristics that are similar to cooked fresh meat. The development of such a product will require a combination of preservatives. Several proposed preservatives that may do this either individually or in combination include nisin, sodium lactate, glucono-delta-lactone and ethylenediaminetetraacetic acid (EDTA).

As previously mentioned, during fermentation lactic acid bacteria produce lactic acid and antimicrobial agents generally classified as bacteriocins. The direct application of lactic acid or a lactate salt and a bacteriocin may be useful in developing a shelf-stable nonfermented meat product. Because of its acidic nature lactic acid incorporated into a meat product will reduce pH, oxidize myoglobin and darken color. However, lactate salts have been reported to exhibit antimicrobial activity without changing the product's physical characteristics (Shelef, 1994). Therefore, a lactate salt such as sodium lactate may be a potential preservative for a shelf-stable meat product.

Many bacteriocins have been isolated and identified. The most studied, best characterized and only bacteriocin with a wide commercial availability in the U.S. is nisin. It has been shown to have antimicrobial effects on a wide variety of Gram-positive bacteria (Delves-Broughton, 1990).

Gluconic acid is produced by the fermentation of D-glucose by microorganisms. Both gluconic acid and its delta-lactone (glucono-delta-lactone) have been shown to act synergistically with both nisin and sodium lactate at inhibiting the growth of various pathogens (Oscroft et al., 1990; Qvist et al., 1994; Zepeda et al., 1994). Glucono-delta-lactone has been shown to be a useful acidulant in cured sausage production. When used at 0.5% of a sausage formulation it will lower the sausage pH by 0.5-0.6 of a pH unit (Haymon, 1981). If glucono-delta-lactone is used in a nonfermented meat product at 0.5% the slight pH reduction will probably have little or no effect on the physical characteristics of the product.

Ethylenediaminetetraacetic acid is a chelating agent that expresses antioxidant and antimicrobial action. Its antimicrobial activity alone in foods is slight, however, EDTA has been shown to have synergistic effects with lysozyme (Moustafa and Collins, 1969; Payne et al., 1994), nisin (Stevens et al., 1991) and potassium sorbate (Robach and Stateler, 1980). The antimicrobial synergism and antioxidant activity exhbited by EDTA may act to enhance the microbial and sensory shelf-life of a cooked nonrefrigerated, noncured meat product.

The effectiveness of nisin, sodium lactate, glucono-delta-lactone, EDTA and their combinations as preservatives needs to be evaluated. This should be done on a broad range of foodborne pathogens in order to obtain accurate and meaningful results. Pathogens were selected on two bases: 1) their pathogenicity to humans and 2) their presence in livestock and resulting presence in meat and meat products. Important pathogens include *Escherichia coli* O157:H7, *Salmonella* spp., *Yersinia enterocolitica*, *Listeria monocytogenes* and *Staphylococcus aureus*. The selected pathogens encompass Gram-negative, Gram-positive and psychrotrophic organisms. Resistance to environmental stresses varies with each organism. The modes of pathogenesis represent both intoxication and infection.

The purpose of this research was to develop a shelf-stable, nonfermented, noncured, restructured pork product. This involved performing experiments to evaluate the antimicrobial activity of nisin, sodium lactate, glucono-delta-lactone, disodium EDTA and their combinations as preservatives in meat products. This was done by investigating their effectiveness as antimicrobial agents on selected foodborne pathogens in cooked meat media and utilizing the most effective combinations of antimicrobial agents in a restructured pork product. The study was divided into four sections: 1) the effects of sodium lactate, glucono-delta-lactone, disodium EDTA, nisin and their combinations on the growth of *E. coli* O157:H7; 2) the effects of sodium lactate, glucono-delta-lactone, nisin and their combinations on the growth of selected foodborne pathogens; 3) the effects of sodium lactate, glucono-delta-lactone, disodium EDTA and their combinations on the growth of *E. coli* O157:H7 and *Salmonella typhimurium*; and 4) the utilization of combinations of sodium lactate and glucono-delta-lactone in nonrefrigerated and refrigerated restructured pork rolls.

CHAPTER 2
LITERATURE REVIEW

Preservatives of Interest

Nisin

The use of nisin and other bacteriocins in food has been thoroughly reviewed (Delves-Broughton, 1990; Hurst and Hoover, 1993; Hansen, 1994; Muriana, 1996; McMullen and Stiles, 1996). Delves-Broughton (1990) focused on nisin's structure, antimicrobial properties and use in food products. Hansen (1994) discussed the structure, antimicrobial mode of action and genetic programing for the biosynthesis of nisin and subtilin. He also developed a rational design and construction to mutate subtilin and enhance its chemical and antimicrobial properties. Hurst and Hoover (1993) discussed nisin's chemical and antimicrobial properties, mode of action, applications, toxicity and legal status. Muriana (1996) addressed the applications of bacteriocins in food, development of bacteriocin resistance and the mode of action of bacteriocins as they applied to controlling *Listeria* spp. in food. McMullen and Stiles (1996) focused on the selection of bacteriocin-producing lactic acid bacteria for biopreservation of meats and research utilizing these organisms. In this review, nisin's chemical structure, antimicrobial activity and mode of action will be discussed. Methods of applying bacteriocins to meat will also be addressed.

Chemical characteristics

Nisin is a ribosomally synthesized peptide that has antimicrobial activity against many food spoilage and pathogenic bacteria. It is produced by the fermentation of a modified milk medium by certain strains of *Lactococcus lactis*. The nisin molecule normally occurs as a dimer with a molecular weight of 7000 daltons. It consists of 34 amino acids and possesses amino and carboxyl end groups and five thioether bonds that form internal rings. The amino acids include L-amino acids and the unusual S-amino acids dehydroalanine, dehydrobutyrine, lanthionine and β-methylanthionine (Delves-Broughton, 1990; Hurst and Hoover, 1993). The presence of lanthionine gives rise to the classification of lantibiotics which includes nisin, subtilin, valinomycin, Gramicidin S., variacin and other bacteriocins produced by food grade bacteria (Hansen, 1994; Pridmore et al., 1996).

The nisin molecule is acidic in nature, being more stable and more soluble when subjected to acidic conditions. In dilute HCl solutions and pH 2.5, its solubility is 12% and there is no loss of activity when autoclaved. The solubility decreases to 4% at pH 5.0, with 40% of its activity being lost during autoclaving. At neutral pH, nisin is practically insoluble and more than 90% of its activity is lost upon autoclaving (Delves-Broughton, 1990). Studies on the effects of processing temperature and pH on nisin stability in different foods have shown a 25-50% destruction of added nisin when heated for 3 min at 121°C, at pH 3.3-4.5 or 6.1-6.9. Large molecules from milk and broth have been reported to protect nisin from heat inactivation, while meat particles enhance inactivation (Hurst and Hoover, 1993).

Toxicity studies using nisin levels in excess of what would be used in foods have shown it to be virtually nontoxic. The median lethal dose (LD_{50}) of nisin to rats and kittens is approximately 7 g/Kg, which is similar to that of common salt (Hurst and Hoover, 1993). Nisin is rapidly inactivated by the digestive enzymes in the intestine and cannot be detected in human saliva 10 min after consuming liquid containing nisin. There is no evidence of allergic reactions to nisin. Microbiological studies have not shown cross resistance in microorganisms that may affect the therapeutic effects of antibiotics. These characteristics suggest that nisin is destroyed when consumed, leaving no potentially toxic residues in the consumer (Delves-Broughton, 1990).

Antimicrobial activity

Nisin exhibits antimicrobial activity toward *Clostridium botulinum, Listeria monocytogenes, Listeria ivanovii, Listeria innocua, Staphylococcus aureus, Streptococcus lactis* and *Bacillus licheniformis* in microbiological media, dairy and fish products and on meat surfaces (Bell and De Lacy, 1985; Somers and Taylor, 1987; Benkerroum and Sandine, 1988; Chung et al., 1989; Taylor et al., 1990; Harris et al., 1991; Motlagh et al., 1991; Stevens et al., 1991). Nisin does not inhibit Gram-negative bacteria such as *Yersinia enterocolitica, Aeromonas hydrophila, Escherichia coli, Salmonella anatum, Salmonella typhimurium, Serratia marcescens* and *Pseudomonas aeruginosa* (Chung et al., 1989; Delves-Broughton, 1990; Motlagh et al., 1991).

Nisin's antimicrobial activity is affected by temperature, pH and the presence of other antimicrobial agents. Thomas and Wimpenny (1996) investigated the effect of simultaneous variations in temperature, pH and NaCl concentration on the inhibitory effect of nisin against *L. monocytogenes* and *S. aureus*. Nisin's inhibition of *S. aureus* was synergistically enhanced by low temperatures and high NaCl concentrations. Action against *L. monocytogenes* was increased with higher NaCl concentrations, but temperature effects were not as apparent. Between pH 7.9 and 5.0, reduced pH appeared to increase nisin's effectiveness against both organisms. At pH 4.5 to 5.0 and 20 to 25°C both organisms exhibited resistance to nisin and NaCl.

Oscroft et al. (1990) observed the effects of incubation temperature, pH, nisin and organic acids on the growth of thermally stressed *Bacillus* spores (*B. cereus* CRA 193, *B. licheniformis* CRA 219, *Bacillus* spp. CRA 220, 190 and 218) in trypticase soy broth. They observed no growth from heat stressed spores at 12°C storage and media pH below 5.7. However, pH did not impede spore growth at 20 or 30°C. The addition of 1000 International Units (IU)/ml of nisin inhibited spore outgrowth for more than 43 days at 10^4/ml inoculum level, 65°C stress temperature, 6.0 pH and 30°C storage temperature. At 500 IU/ml, nisin inhibited spore outgrowth for more than 43 days at 10^6/ml inoculum level, 65°C stress temperature, 5.7 pH and 30°C storage temperature.

Cutter and Siragusa (1995a, 1995b) evaluated the antimicrobial effectiveness of nisin (50 μg/ml) alone or in combination with chelating agents [50 mM EDTA, 500 mM lactate, 100 mM citrate or 1% sodium hexametaphosphate (HMP)] against *S. typhimurium* and *E. coli* O157:H7. The treatments were applied in 3-[N-morphilino] propanesulfonic acid (MOPS) or phosphate buffered saline (PBS) and incubated for 60 min at 37°C or 30 min at 5°C. For *E. coli* O157:H7, when used alone nisin, lactate and citrate reduced counts by 3.41, 1.8 and 1.47 $\log_{10}$ CFU/ml, respectively. EDTA and HMP did not reduce

($P > 0.05$) counts. The combination of nisin and EDTA reduced *E. coli* O157:H7 numbers by 5.42 $\log_{10}$ CFU/ml and was more effective ($P \leq 0.05$) than either nisin or EDTA alone. Combining nisin with lactate, citrate or HMP did not affect ($P > 0.05$) nisin's antimicrobial activity toward *E. coli* O157:H7. For *S. typhimurium*, nisn used alone reduced counts by 2.31 $\log_{10}$ CFU/ml, while lactate, citrate, EDTA and HMP did not affect ($P > 0.05$) counts. Combinations of nisin with lactate, citrate and EDTA reduced *S. typhimurium* counts by 4.25, 4.06 and 4.53 $\log_{10}$ CFU/ml, respectively and were more effective ($P \leq 0.05$) than nisin alone. Combining nisin with HMP did not effect ($P > 0.05$) nisin's antimicrobial activity (Cutter and Siragusa, 1995a). The treatments were also applied to lean beef tissue with *S. typhimurium* or *E. coli* O157:H7 attached to the surface and incubated at 4°C for up to 3 days. Combinations of nisin with EDTA or lactate significantly ($P \leq 0.04$) reduced the numbers of *S. typhimurium* and *E. coli* O157:H7 by 0.33 to 0.42 $\log_{10}$ CFU/cm^2 when compared to untreated tissue. Nisin, alone or in combination with citrate or HMP did not affect microbial numbers (Cutter and Siragusa, 1995b).

Combinations of nisin (400 IU/ml) with sodium nitrite (0.0125%), potassium sorbate (0.3%), sodium lactate (4%) and/or polyphosphate (0.5%) in beef heart infusion broth (pH 5.5) exhibited bacteriostatic to bactericidal effects on *L. monocytogenes* at 4°C. In the same system, nisin exhibited an immediate 1 $\log_{10}$ reduction in population with no long term inhibitory effects (Buncic et al., 1995).

Stevens et al. (1991) exposed Gram-negative bacteria to 50 μg of nisin per ml and 20 mM disodium ethylenediaminetetraacetic acid (EDTA) at 37°C for 1 hr. They observed at least a 3.2 $\log_{10}$ cycle reduction for *Salmonella* spp., *Enterobacter aerogenes*, *Shigella flexneri*, *Citrobacter freundii* and *E. coli* 0157:H7. However, individually neither nisin nor EDTA reduced microbial counts by greater than 1 $\log_{10}$.

Stevens et al. (1992b) evaluated the antimicrobial activity of nisin toward *Salmonella* spp. and other Gram-negative bacteria. The effects of chelating agents, nisin concentration, incubation temperature and protein interference on nisin's activity were also examined. Each organism was exposed to nisin/chelator combinations in cell buffer solutions at 37°C and pH 6.5 for 60 min unless the effects of incubation temperature were being examined. The effects of chelating agents were evaluated by combining nisin (50 μg/ml) with 20 mM of EDTA, ethylenebis (oxyethylene-nitrilo) tetraacetic acid (EGTA), citric acid monohydrate or sodium phosphate dibasic. The mean log reduction across all test organisms was 5.9, 3.8, 5.1 and 4.0 for EDTA, EGTA, citric acid and phosphate, respectively. These results indicated that EDTA was the most effective chelator for enhancing nisin's antimicrobial activity toward Gram-negative bacteria. The combination of nisin and EDTA reduced *Salmonella* spp. counts by 5.3 to 6.7 log cycles and *E. coli* O157:H7 counts by 6.9 log cycles. The effects of nisin concentration were determined by combining 10, 25, 50 or 100 μg/ml nisin with 20 mM EDTA. The mean log reductions across nisin levels were 2.1, 3.4, 5.0 and 5.0 for 10, 25, 50 and 100 μg/ml nisin, respectively. This trend reveals that nisin's effectiveness increases as concentration increases up to 50 μg/ml. The effect of incubation temperature was examined by exposing *Salmonella* spp. to 50 μg/ml nisin and 20 mM EDTA at various incubation temperatures. The mean log reductions across incubation temperatures were 2.0, 3.4, 4.4, 5.0 and 5.0 for 4, 20, 30, 37 and 42°C, respectively. Increasing incubation temperature increased nisin's effectiveness. The effect of protein interference was evaluated by comparing the survival of *Salmonella* spp. in cell buffer solutions containing 50 µg/ml nisin and 20 mM EDTA with and without 1 mg/ml bovine serum albumin (BSA). The mean log reductions were 4.9 and 4.6 for nisin/EDTA solutions with and without BSA, respectively, indicating that BSA did not interfere appreciably with nisin's antimicrobial activity toward Gram-negative bacteria.

Maltol and ethyl maltol have also been reported to increase the susceptibility of *E. coli* cells to nisin (Schved et al., 1996). The proposed mechanism is that maltol and ethyl maltol chelate Mg^{2+} and Ca^{2+} in the lipopolysaccharide membrane and expose the cell membrane to nisin.

With the utilization of bacteriocins in food, there is growing concern about the potential development of bacteriocin resistance among bacteria (Muriana, 1996). Harris et al. (1991) reported that *L. monocytogenes* Scott A and ATCC 19115 formed nisin resistant mutations at frequencies of 10^{-6} to 10^{-8}, indicating that widespread nisin application in foods could result in nisin resistant variants. Fundamental changes occur in the membrane structure of *L. monocytogenes* as a resistance response to nisin (Ming and Daeschel, 1993). The resistance response was later correlated to reductions in the total levels of three phospholipids (phosphatidylglycerol, diphosphatidylglycerol and bisphosphatidylglyceryl phosphate) in the cell membrane (Ming and Daeschel, 1995). The potential of developing bacteriocin resistance may be reduced by using a combination of bacteriocins with different mechanisms of action (Muriana, 1996).

Mode of action

Research on nisin's mode of action on vegetative cells indicates that nisin disrupts the cytoplasmic membrane resulting in the leakage of essential cellular material or cell lysis (Delves-Broughton, 1990). Nisin acts as a cationic surface active detergent and adsorption to the microbe appears to be the first step of its action. Its adsorption to sensitive cells is highly pH dependent. At 150 IU/ml, nisin's adsorption to group B *Streptococcus* was reported to be 100, 69 and 45% at pH 6.5, 5.5 and 4.5, respectively. At 1500 IU/ml, nisin inhibited 50% of the enzymes involved with peptidoglycan synthesis in *Bacillus stearothermophilus* and *E. coli* (Hurst and Hoover, 1993).

Stevens et al. (1992a) reported that the removal of the outer cell membrane on *S. typhimurium* either by chemical agents or physiological mutations increased its sensitivity to nisin, indicating that nisin's site of action in Gram-negative bacteria may be the cytoplasmic membrane. Carneiro de Melo et al. (1996) investigated nisin's affect on oxygen consumption by *E. coli* and *S. aureus*. Nisin stimulated oxygen consumption in both organisms indicating a protonophore mode of action. Nisin also stimulated oxygen consumption by *E. coli* cells that had been osmotically shocked to disrupt the outer cell membrane, confirming the cytoplasmic membrane as nisin's site of action for Gram-negative bacteria.

The effects of nisin on the proton motive force (PMF) of *L. monocytogenes* and *C. sporogenes* have been investigated. Nisin, at 5 μg/ml or greater resulted in the complete loss of PMF in *L. monocytogenes* Scott A cells at pH 5.5 and 7.0. The action was both time and concentration dependent. The PMF of *L. monocytogenes* V7, FRI-LM

103M, F5069 and ATCC 19115 cells was depleted by 2.5 μg/ml nisin at pH 6.5 (Bruno et al., 1992). Similarly, nisin (250 μg/ml) depleted the ATP and dissipated the PMF of *C. sporogenes* PA3679. These results indicated that nisin increased the permeability of the cytoplasmic membrane to ions (Okereke and Montville, 1992). Winkowski et al. (1996) summarized the theory of nisin's action on the PMF through a poration mechanism. The widely accepted "barrel-stave" model of poration suggests that the nisin monomers ("the staves") bind to the membrane, insert and aggregate within the membrane to form a pore ("the barrel"). The hydrophobic regions of the nisin molecule become perpendicularly oriented into the membrane leaflets and the hydrophilic regions face inward forming the lumen of the pore. The development of the pore allows a free exchange of ions between the cell and its environment, which ultimately results in the death of the cell.

Winkowski et al. (1994) reported that nisin induced ATP efflux, rapidly reduced the intracellular ATP concentration and rapidly dissipated the PMF of *L. monocytogenes*. ATP efflux depended on nisin concentration and saturation kinetics, suggesting that nisin breaches the permeability barrier in a manner consistent with "pore" formation. Abee et al. (1994) evaluated the action of nisin Z, a natural variant of nisin, on *L. monocytogenes*. Nisin Z caused immediate loss of cell K^+, depolarization of the cytoplasmic membrane, inhibition of respiratory activity, and hydrolysis and partial efflux of cellular ATP. The action was optimal at pH 6.0. Nisin Z's action was reduced at low temperatures and by di- and trivalent cations and inhibited by lanthanide gadolinium (Gd^{3+}).

Nisin's action against spores has been reported to be sporicidal rather than sporistatic. Nisin is believed to inhibit germination at the pre-emergent swelling stage, while sporistatic preservatives act after the cell has shed the spore wall (Delves-Broughton, 1990)

Methods of applying bacteriocins to meat

Nisin's application as a food preservative was first mentioned by Hirsch et al. (1951), who observed that nisin-producing starter cultures inhibited clostridial gas formation in cheese. Nisin and other bacteriocins have been experimentally applied indirectly to food products via bacteriocin-producing cultures as biocontrol microorganisms in nonfermented foods and as starter cultures in fermented foods and directly to food products by adding bacteriocin-containing fermentates or purified bacteriocins (Muriana, 1996).

The main advantage of utilizing bacteriocin producing microorganisms as biocontrol or fermentation starter cultures in meat is that most organisms currently being used have GRAS status with the United States Food and Drug Administration (USFDA). This allows the indirect addition of bacteriocins to the meat product via natural growth processes. However, the production of bacteriocins is dependent upon the growth of the organism. Therefore, temperature, pH, packaging, preservatives and availability of nutrients will affect the quantity of bacteriocin produced and the shelf-life of the product.

Studies using bacteriocin-producing cultures as biocontrol microorganisms in nonfermented meats have shown variations in antilisterial activity depending upon the initial levels of culture and storage temperature. Berry et al. (1991) observed that bacteriocin producing *Pediococcus acidilactici* JD1-23 at inoculum levels of 10^7 CFU/g inhibited the growth of *L. monocytogenes* for 60 days while levels of 10^4 CFU/g extended the lag phase by approximately 20 days on frankfurters inoculated and stored at 4°C. *P. acidilactici* JD1-23 also inhibited the growth of *L. monocytogenes* under aerobic and anaerobic storage at 15°C. The antilisterial activity of *P. acidilactici* JD1-23 was only partially attributed to bacteriocin production because *P. acidilactici* JD-M (a bacteriocin nonproducing strain) also exhibited antilisterial activity to a lesser extent than the JD1-23.

Similarly, Degnan et al. (1992) evaluated *P. acidilactici* JBL1095 (pediocin AcH producer) and LB42 (pediocin nonproducer) for their antilisterial activity in packages of all beef wieners stored at 4 and 25°C. There were no significant changes in *L. monocytogenes* or *P. acidilactici* numbers for 72 days of storage at 4°C. Likewise, there was no change in pH. Therefore, it was concluded that at 4°C there was insufficient growth to result in the production of acid or bacteriocin. At 25°C, LB42 inhibited *L. monocytogenes*, while JBL1095 was bactericidal.

In another biocontrol system, Winkowski et al. (1993) inoculated vacuum sealed beef cubes, beef cubes in gravy and beef cubes in gravy containing glucose with *Lactobacillus bavaricus* at 10^3 or 10^5 CFU/g and with *L. monocytogenes* at 10^2 CFU/g and stored them at 4 and 10°C. *L. bavaricus* inhibited the growth of *L. monocytogenes* in all systems and was bactericidal in systems containing gravy. Inhibition was more effective with 10^5 CFU/g *L. bavaricus* and 4°C storage than the other inoculum level and storage temperature combinations. Bacteriocin was detected and inhibition could not be attributed to acidification. In a similar system, bacteriocin and nonbacteriocin producing strains of

L. lactis, *Lactobacillus plantarum* and *Pediococcus pentosaceus* prevented toxin production by heat shocked *C. botulinum* spores in a gravy-like media containing 0.5% glucose and incubated at 15°C (Crandall and Montville, 1993). However, bacteriocin and nonbacteriocin producing strains of *P. pentosaceus* did not prevent toxin production by *C. botulinum* in minimally heat-treated, vacuum-packaged *sous vide* type beef with gravy stored at 4 and 10°C (Crandall et al., 1994).

Berry et al. (1990) evaluated the effects of bacteriocin producing and nonbacteriocin producing *Pediococcus* on the survival of *L. monocytogenes* in fermented semidry summer sausage. During fermentation, bacteriocin producing *Pediococcus* reduced *L. monocytogenes* by 2 $\log_{10}$ CFU/g, while less than a 1 $\log_{10}$ CFU/g reduction was observed for nonbacteriocin producing *Pediococcus*. *Listeria* was intermittently recovered after heating to an internal temperature of 64.4°C, indicating that the bacteriocin was not effective at inhibiting *L. monocytogenes* that survived the heating process. Foegeding et al. (1992) and Baccus-Taylor et al. (1993) have also shown that the utilization of pediocin producing strains of *P. acidilactici* enhanced the antilisterial effects of the fermentation process for both dry fermented pork sausage and chicken summer sausage.

The direct application of bacteriocins to a food allows the manufacturer to control the concentration of the bacteriocin in/on a given food. The bacteriocins discussed have had immediate antimicrobial effects. However, when they were applied to meat with high microbial loads they only exhibited short term inhibitory effects. Currently, the only bacteriocin approved by the USFDA as a direct food additive is nisin (Muriana, 1996).

Studies applying bacteriocins directly to meat have focused on their use in meat slurries or bacteriocin dips to control microbial growth on meat surfaces. Nisin has had mixed results in meat slurries. Rayman et al. (1983) reported that 500 ppm (0.05%) nisin and 60 ppm (0.006%) nitrite did not inhibit the outgrowth of *C. botulinum* in pork slurries at pH 5.8. At pH 5.1, 300 ppm nisin completely inhibited spore outgrowth and at pH 5.5 it was ineffective.

Motlagh et al. (1992) evaluated the effectiveness of pediocin AcH in reducing the growth of *L. monocytogenes* strains and *L. ivanovii* in sterile ground beef, sausage mix, cottage cheese, ice cream and reconstituted dry milk suspended in water. The maximum bactericidal action of pediocin AcH occurred within 1 hr of inoculation and was not interfered with by the food. Pediocin AcH was most effective when pediocin was at high concentrations and initial *Listeria* levels were low. Cells that survived the pediocin treatment multiplied in the presence of residual pediocin AcH during storage at 4 and 10°C.

Chung et al. (1989) performed a series of experiments to evaluate the effects of nisin (10^4 IU/ml) on the attachment and growth of various Gram-positive and Gram-negative bacteria attached to meat. After 10 min at room temperature there was a 1 to 1.9 $\log_{10}$ reduction in Gram-positive cells attached to the meat surface depending on the organism (*S. lactis, S. aureus* and *L. monocytogenes*). For the Gram-negative organisms, *S. typhimurium, S. marcescens* and *P. aeruginosa*, nisin did not affect attachment. At room temperature, nisin initially caused a 2 $\log_{10}$ CFU/4 cm^2 reduction in *S. aureus* and *L. monocytogenes* attached to meat. However, during storage there was rapid growth, with the nisin treated samples exhibiting the same number of organisms as the controls after 2 to 3 days of incubation. Under refrigerated storage (5°C), there were similar initial reductions in numbers and the nisin treatments exhibited similar growth curves as the controls.

In similar experiments, El-Khateib et al. (1993) investigated the effects of nisin and pediocin on the attachment of *L. monocytogenes* to meat cubes. Nisin, at 4 x 10^4 IU/ml, caused an immediate 1 $\log_{10}$ reduction in viable cells. Nisin did not exhibit delayed effects during storage. Similarly, a crude preparation of pediocin PO2 [3.2 x 10^3 arbitrary units (AU)/ml] reduced *L. monocytogenes* numbers by 0.6 $\log_{10}$ CFU/cube immediately with no delayed effects during storage.

Nielsen et al. (1990) evaluated the inhibitory effects of a bacteriocin containing fermentate from *P. acidilactici* on *L. monocytogenes* associated with fresh meat. They observed that 500 to 5,000 AU/ml reduced the numbers of attached *L. monocytogenes* by 0.5 to 1.5 $\log_{10}$ cycles when a high (8 x 10^5 CFU/4 cm^2) inoculation was used. The same pediocin levels resulted in a 1.3 to 2.2 $\log_{10}$ reduction when initial *L. monocytogenes* levels were low (8 x 10^3 CFU/4 cm^2). The antilisterial activity occurred within the first two min of exposure. At 1,000 and 5,000 AU/ml inhibitory effects were observed for 21 days at 5°C.

Fang and Lin (1994) dipped cooked pork loins into nisin solutions (0, 10^3 and 10^4 IU/ml) and inoculated the tenderloins with 3.18 and 3.24 $\log_{10}$ CFU/g *L. monocytogenes* and *Pseudomonas fragi*, respectively. After inoculation, the tenderloins were packaged under 100 and 80% CO_2 and an atmospheric gas control and stored at 4 and 20°C. Nisin was ineffective against *P. fragi* under all storage conditions. Initially nisin exhibited a 2 $\log_{10}$ reduction on *L. monocytogenes* at both 10^3 and 10^4 IU/ml and under all storage conditions. At 10^3 IU/ml nisin and 20°C storage, *L. monocytogenes* grew after 1 day of storage. At 10^4 IU/ml nisin was bactericidal with no growth occurring during 30 and 10 days of storage at 4 and 20°C, respectively.

Mahadeo and Tatini (1994) incorporated nisin into water used for scalding tturkeys (scald water), at 100 IU/ml and evaluated its effectiveness against *L. monocytogenes* directly suspended in the water or attached to turkey skin suspended in the water. With initial levels of 5.5 $\log_{10}$ CFU/ml, nisin reduced the number of *L. monocytogenes* directly suspended in the scald water by 1 $\log_{10}$ CFU/ml. Nisin and heat (52°C for 3 min) reduced numbers in the scald water by 3.5 $\log_{10}$ CFU/ml. With initial levels of approximately 5.7 $\log_{10}$ CFU/ml treatment with nisin or nisin and heat reduced the numbers of *L. monocytogenes* attached to turkey skin suspended in scald water by 1 $\log_{10}$ CFU/ml. Chilling for 48 hr at 4°C further reduced numbers by another 1 to 2 $\log_{10}$ cycles.

Lactates

Smulders et al. (1986) and Guerro and Taylor (1994) reviewed the use of lactic acid as a meat surface decontaminant. Smulders et al. (1986) focused on lactic acid's physical properties, natural occurrence in foods and use as a meat decontaminant. Guerro and Taylor (1994) discussed the changes in meat caused by microbial contamination and the decontamination of meat with either lactic acid or lactic acid producing bacteria. Lactic acid was also included in a review of organic acids by Doores (1993), who discussed the toxicology, antimicrobial properties and regulatory status of lactic acid and the general mode of action for organic acids. Shelef (1994) addressed the physical properties of lactic acid and lactate salts, their antimicrobial activity and their probable mechanisms of antimicrobial activity. This review of lactates will focus on their chemical characteristics, antimicrobial activity, mode of action and applications in/on meat products.

Chemical characteristics

Lactic acid is one of the most widely distributed acids in nature, occurring in the blood, muscles and organs of man and animals. It is a derivative of propionic acid, with a molecular formula of $C_3H_6O_3$ and molecular weight of 90.08. The dissociation constant (pKa) for lactic acid at 25°C has been reported to be 3.76 (Merck & Co., Inc., 1989) or 3.86 (Segel, 1976). At 25°C, 1 and 10% solutions of lactic acid exhibit pH values of 2.28 and 1.75, respectively (Shelef, 1994).

Lactic acid can be self-esterified by heating at 120°C for ten hr to form lactic acid lactate, $CH_3CH(OH)COOCH(CH_3)COOH$. Self-esterification also occurs at room temperature, lactic acid lactate forms at a concentration of 4.2% in a 6.3% lactic acid solution, 12.3% in a 55% lactic acid solution and 33.8% in an 88% lactic acid solution (Shelef, 1994).

The USFDA recognizes lactic acid as a generally recognized as safe (GRAS) ingredient for direct use in human foods, with the exception of infant foods and formula. There are no limitations to its use in food other than current good manufacturing practices. Lactic acid can be used as an antimicrobial agent, curing and pickling agent, a flavor enhancer, a flavoring agent or adjuvant, a pH control agent or a solvent and vehicle (USFDA, HHS, 1995).

Sodium, potassium and calcium salts of lactic acid are commercially available for food applications, as mixtures with water containing 60-80% of the respective lactate. Lactates are useful in foods because they possess similar characteristics as lactic acid, yet have a neutral pH and their application does not reduce pH.

The USFDA recognizes sodium, potassium and calcium lactates as generally recognized as safe (GRAS) ingredients for direct use in human foods, with the exception of infant foods and formula. There are no limitations to their use in food other than current good manufacturing practices. Sodium, potassium and calcium lactates can be used as emulsifiers, flavor enhancers, flavoring agents or adjuvants, humectants or pH control agents. Calcium lactate has additional applications as firming agent, leavening agent, nutrient supplement, stabilizer and thickener (USFDA, HHS, 1995).

Antimicrobial activity of lactic acid

Sterile beef lean and adipose tissue were inoculated with either *S. typhimurium, E.coli* O157:H7 or *L. monocytogenes* Scott A, and washed with 23°C distilled water or 1% lactic acid (Dickson and Siragusa, 1994). The washed tissues were stored at 5 °C and 26% RH for up to 21 days. The greatest reduction in pathogens occurred in the first 7 days of storage. From 10 to 21 days of storage, the pathogen levels of the lactic acid rinsed lean were approximately 1.2, 0.5 and 2.0 $\log_{10}$ CFU/cm^2 lower than the water-washed lean for *S. typhimurium, E.coli* O157:H7 and *L. monocytogenes*, respectively. Similarly for the adipose tissue, lactic acid washed samples were 3, 2 and 4 $\log_{10}$

CFU/cm^2 lower than the water-washed samples for *S. typhimurium, E. coli* O157:H7 and *L. monocytogenes*, respectively.

Similarly, Podolak et al. (1995 and 1996) reported that inoculated semitendinosus muscle dipped in lactic acid (1%) reduced the counts of *S. typhimurium, E. coli* O157:H7 and *L. monocytogenes* during vacuum packaged storage at 4°C. Podolack et al. (1995) reported that a 15 s dip in 1% lactic acid initially reduced *L. monocytogenes* numbers by 0.40 $\log_{10}$ CFU/cm^2. After 14 days of storage, *L. monocytogenes* numbers were 0.55 $\log_{10}$ CFU/cm^2 less than the control. A 30 s dip in 1% lactic acid reduced *L. monocytogenes* numbers by 1.12 $\log_{10}$ CFU/cm^2. After 14 days, counts were 1.06 $\log_{10}$ CFU/cm^2 lower than the control. Dipping in 1% lactic acid for 15 and 30 s resulted in an initial 0.50 to 0.60 $\log_{10}$ CFU/cm^2 reduction in numbers for both *S. typhimurium* and *E. coli* O157:H7. After 14 days of storage, counts were 0.20 to 0.40 $\log_{10}$ CFU/cm^2 lower than the control.

Podolack et al. (1996) performed similar evaluations on the antimicrobial effects of 1% lactic acid dip (5 s) on *L. monocytogenes* and *E. coli* O157:H7 stored at 4°C. There was an initial 0.50 $\log_{10}$ CFU/cm^2 reduction in *L. monocytogenes* when compared to the control. By day 21, the reduction was only 0.30 $\log_{10}$ CFU/cm^2. From day 30 to 90 the reduction was approximately 0.10 $\log_{10}$ CFU/cm^2. For *E. coli* O157:H7 there was an initial 0.75 $\log_{10}$ CFU/cm^2, which did not change during 21 days of storage. From 30 to 60 days, there was a 0.20 $\log_{10}$ CFU/cm^2. At day 90, there was only a 0.10 $\log_{10}$ CFU/cm^2 reduction in *E. coli* O157:H7 numbers.

Van Netten et al. (1994a, 1994b) investigated the immediate bactericidal effect of lactic acid on pork belly surfaces and in a model meat system consisting of suspended pork skin. Decontamination of mesophilic *Enterobacteriaciae* with 2% lactic acid at 21°C resulted in an immediate lethality of 0.3 and 0.8 $\log_{10}$ CFU/ml in the model meat system and 0.4 and 1.0 $\log_{10}$ CFU/cm^2 after exposure for 30 and 60 s, respectively. Exposing selected pathogens to 2% lactic acid for 30 s at 21°C resulted in immediate lethalities of 2.6, 0.6 and 0.0 $\log_{10}$ CFU/ml in the model system and 2.5, 0.6 and 0.0 $\log_{10}$ CFU/cm^2 on pork belly surfaces for *Campylobacter jejuni*, *S. typhimurium* and *L. monocytogenes*, respectively (Van Netten et al., 1994a).

Utilizing the previously discussed *in vitro* model, Van Netten et al. (1994b) studied the kinetics of the bactericidal effect of lactic acid decontamination on *Salmonella* spp., *C. jejuni* and *L. monocytogenes*. A step-wise increase in pH from 2.6 to 3.5 and 4.0 progressively reduced the bactericidal effect of decontamination. The immediate death of *Salmonella* spp. treated with 2% lactic acid for 30-90 s at 21°C was 0.5-2.0 $\log_{10}$ CFU at pH 2.6, when the pH was increased to 4.0, the immediate death decreased to 0.0 $\log_{10}$ CFU. Similarly, the immediate death of *C. jejuni* was 2.6 to > 5.3 at pH 2.6 and 21°C, when the pH was increased to 4.0 the immediate death decreased to 0.3-1.0 $\log_{10}$ CFU. Increasing the temperature from 21 to 37°C countered the reduced bactericidal effect that occurred with increasing pH. Decontamination with 2.0% lactic acid at 31 or 37°C did not exhibit an immediate bactericidal effect on *L. monocytogenes*.

Brackett et al. (1993) applied warm (20°C) and hot (55°C) acetic, citric and lactic acid sprays (0.5, 1.0 and 1.5% concentrations) to beef sirloin tip slices to evaluate their effectiveness at decontaminating beef inoculated with *E. coli* O157:H7. Acid sprays did not reduce *E. coli* O157:H7 levels by more than 0.30 $\log_{10}$ CFU/g when initial levels were

10^3 or 10^6 CFU/g. Therefore, they concluded that warm and hot acid sprays were ineffective at decontaminating beef of *E. coli* O157:H7.

The acid injury and D-values of *L. monocytogenes* in tryptone broth acidified with 0.3 or 0.5% lactic acid and incubated at 13 or 35°C were determined by Ahamad and Marth (1990). Uninjured cells were identified by enumerating on tryptose agar containing 6% NaCl. The initial counts for the 13°C incubation were 5.5 $\log_{10}$ CFU/ml. After 500 hr in 0.3% lactic acid the levels were 3.5 $\log_{10}$ CFU/ml total *L. monocytogenes* and 1 $\log_{10}$ CFU/ml uninjured cells. After 500 hr in 0.5% lactic acid numbers decreased to 2.0 $\log_{10}$ CFU/ml total *L. monocytogenes* and no uninjured cells. At 35°C, counts at inoculation were 6.0 $\log_{10}$ CFU/ml. The total numbers of *L. monocytogenes* were reduced to 3.0 $\log_{10}$ CFU/ml, with 1 $\log_{10}$ CFU/ml of uninjured cells after 60 hr in 0.3% lactic acid. Similarly, in 0.5% lactic acid total counts were 3.0 $\log_{10}$ CFU/ml with 1.0 $\log_{10}$ CFU/ml of uninjured cells. The D-values were 106 hr for 13°C and 0.3% lactic acid, 95 hr for 13°C and 0.5% lactic acid, 11.2 hr for 35°C and 0.3% lactic acid and 9.5 hr for 35°C and 0.5% lactic acid.

Giannuzzi and Zaritzky (1996) investigated the effects of several organic acids on the growth of *L. monocytogenes* at refrigerated temperatures. They evaluated acid effectiveness as a function of pH, total acid concentration and undissociated acid concentration. An exponential growth rate near 0 $\log_{10}$ CFU/ml/days was observed at pH 5.0 and 0.97 mmol/L undissociated lactic acid (Table 2-1). Further reduction in pH and corresponding increases in the undissociated acid concentration resulted in negative exponential growth rates or bactericidal effects.

Table 2-1. The growth of *Listeria monocytogenes* in trypticase soy broth at various pH values, total lactic acid and undissociated lactic acid concentrations

pH	Total Acid Concentration (mmol/L)	Undissociated Acid Fraction	Undissociated Acid Concentration (mmol/L)	Exponential Growth Rate ($\log_{10}$ CFU/ml/day)	
				4°C	10°C
6.5	1.4	0.0014	0.002	0.30	0.68
6.0	6.6	0.0076	0.005	0.21	0.62
5.0	14.4	0.067	0.97	-0.06	-0.07
4.5	22.2	0.18	4.0	-0.10	-0.06
4.0	37.7	0.42	16.0	-0.41	-0.31
3.5	66.6	0.70	46.0	-1.10	-1.65

Data adapted from Giannuzzi and Zaritzky (1996).

Brackett (1987) evaluated the effects of several acids on the growth and survival of *Yersinia enterocolitica* strains C122-76 and Y7P in tryptic soy broth for 24 hr at 25°C. The effectiveness of each acid was reported on the basis of pH, total acid concentration and undissociated acid concentration. The results for lactic acid are presented in Table 2-2. When the undissociated acid concentration was between 2 and 18 mmol/L there was a rapid increase in the inactivation of both *Y. enterocolitica* strains. The concentration of undissociated lactic acid where activity increased is similar to the concentration that was reported to inactivate *L. monocytogenes* (Giannuzzi and Zaritzky, 1996).

Table 2-2. Inactivation of *Yersinia enterocolitica* C122-76 and Y7P after 24 hr of incubation at 25°C in tryptic soy broth acidified with lactic acid at various pH values, total acid and undissociated acid concentrations

pH	Total Acid Concentration (mmol/L)	Undissociated Acid Concentration (mmol/L)	Change in Counts ($\log_{10}$ CFU/ml)	
			C122-76	Y7P
7.1	0	0	0	0
5.0	25	2	0	0
4.2	50	18	-6.5	-5.6
4.0	75	35	-8.8	-7.4
3.6	100	62	-8.8	-8.5

Data adapted from Brackett (1987).

Little et al. (1992) also investigated the effects of pH, acidulant and temperature on the survival of *Y. enterocolitica* in tryptone soya broth. At pH 3.0, lactic acid was bactericidal to *Y. enterocolitica* (10^6 CFU/ml) within 24 hr of incubation at 0, 4, 10 and 23°C. At pH 3.5, lactic acid exhibited D-values of 0.5, 0.3 and 0.2 days at 0, 4 and 10°C, respectively. At 23°C, there were no viable *Y. enterocolitica* detected after 24 hr. At pH 4.0, lactic acid D-values were 11.1, 3.4, 1.3 and 0.4 days at 0, 4, 10 and 23°C, respectively. At pH 4.5, lactic acid D-values were 14.8, 5.2, 1.9 and 0.6 days at 0, 4, 10 and 23°C, respectively. The antimicrobial activity of lactic acid toward *Y. enterocolitica* increased as pH decreased and as incubation temperature increased. At pH ≤ 4.0, lactic acid was less effective than acetic acid and more effective than citric and sulfuric acids. At pH 4.5 and 4 to 23°C, lactic acid was more effective than acetic, citric and sulfuric acids.

Similarly, Abdul-Raouf et al. (1993) evaluated the survival of *E. coli* O157:H7 in ground, roasted beef as affected by pH, acidulant and temperature. They utilized 10% acetic, citric or lactic acid to acidify 125 ml of 20% beef slurry from a pH of 5.98 to 5.40, 5.00 and 4.7. The resulting total acid concentration, undissociated acid fraction, undissociated acid concentration and exponential growth rates for lactic acid are presented in Table 2-3. The slurries were inoculated with *E. coli* O157:H7 and incubated at 5, 21 or 31°C for 24 hr. At pH 4.7 and 5°C, lactic acid reduced *E. coli* numbers by 2 $\log_{10}$ CFU/ml when compared to the control. At pH 4.7 and 21°C, lactic acid exhibited bacteriostatic effects by reducing *E. coli* O157:H7 numbers by 1 $\log_{10}$ CFU/g from initial levels while the control exhibited growth to 10^9 CFU/ml. At pH 4.7 and 30°C, lactic acid resulted in 10^6 CFU/ml while the control was 10^9 CFU/ml after 24 hr. At other pH and temperature combinations lactic acid exhibited less inhibitory activity than at pH 4.7. The concentration of undissociated acid at which lactic acid exhibited antimicrobial activity toward *E. coli* O157:H7 was 2.13 mmol/L and is similar to the concentration of undissociated lactic acid that inhibited the growth of *L. monocytogenes* as reported by Giannuzzi and Taylor (1996).

Glass et al. (1992) observed that when trypticase soy broth was acidified with lactic acid to a pH of 4.5 and incubated at 37°C there was a 4 $\log_{10}$ CFU/ml reduction in the numbers of *E. coli* O157:H7 within 14 days. However, at pH 4.6 or greater *E. coli* O157:H7 survived and grew. They also reported that *E. coli* O157:H7 inoculated into commercial sausage batter at 4.8 x 10^4 CFU/g survived fermentation to pH 4.8, drying to a moisture:protein ratio of $\geq$ 1.9 and storage at 4°C for 2 months. Other investigations have indicated that *E. coli* O157:H7 and *Salmonella* spp. are capable of survival and growth under acidic conditions (Leyer and Johnson, 1992; Brackett et al., 1993; Leyer and Johnson, 1993; Arnold and Kaspar, 1995; Benjamin and Datta, 1995; Conner and Kotrola, 1995; Leyer et al., 1995).

Table 2-3. The growth of *Escherichia coli* O157:H7 in a 20% slurry of ground, roasted beef at various pH values, total lactic acid and undissociated lactic acid concentrations

pH	Total Acid Concentration (mmol/L)	Undissociated Acid Fraction	Undissociated Acid Concentration (mmol/L)	Change in Numbers[a] (CFU/ml)		
				5°C	21°C	30°C
5.4	4.4	0.0280	0.123	±	+	+
5.0	8.9	0.0676	0.602	±	+	+
4.7	16.9	0.1263	2.134	-	-	+

[a] + increasing numbers, ± no change in numbers, - decreasing numbers.
Data adapted from Abdul-Raouf et al. (1993).

Conner and Kotrola (1995) evaluated the effect of pH reduction on the growth and survival of *E. coli* O157:H7 in trypticase soy broth containing 0.6% yeast extract with six different organic acids. Overall they observed that *E. coli* O157:H7 has the ability to survive in acidic conditions (pH ≥ 4.0) for up to 56 days. Survival was affected by acidulant and temperature. Preliminary studies with lactic acid exhibited bactericidal effects at pH 5.0 and 10°C and pH 4.0 and 25 or 37°C. In a 56 day study, *E. coli* O157:H7 survived at 4 and 10°C and grew at 25°C in the presence of lactic acid at a pH of 4.7, a total acid concentration of 33 mmol/L and an undissociated acid concentration, of 1 mmol/L.

Leyer et al. (1995) adapted *E. coli* O157:H7 to acid by culturing for one to two doublings at pH 5.0. The acid adapted cells exhibited a 1 to 4 $\log_{10}$ greater percent survival than nonacid adapted cells after 60 min in E buffer (medium E without the glucose supplementation) acidified with 125 mM lactic acid, pH 3.85. The acid adapted cells also exhibited higher survival rates than their nonacid adapted counterparts in sausage during 14 hr of fermentation (pH 5.6 to 4.4 and 32°C) and after 100 hr in shredded hard salami (pH 5.0 and 5°C). In apple cider (pH 3.46 and 6°C) no viable cells were detected after 25 hr for nonacid adapted cultures and after 125 hr for acid adapted cultures.

Similarly, Arnold and Kaspar (1995) observed that the acid tolerance (in synthetic gastric fluid, pH 1.5) of *E. coli* O157:H7 strains was enhanced when the cells were in the log-phase of growth under starvation conditions and in the stationary phase of growth. Benjamin and Datta (1995) observed that *E. coli* O157:H7 (ATCC 43896) did not lose viability for at least 5 hr at pH 3.0 or 2.5 and 37°C. Other enterohemorrhagic *E. coli* (EHEC) strains exhibited high levels of acid tolerance, which was dependent upon growth phase and the pH of the growth media.

Leyer and Johnson (1992) adapted *Salmonella typhimurium* to acid by culturing for one or two doublings at pH 5.8. Acid adapted cells exhibited increased resistance to inactivation by 125 mM lactic, propionic or acetic acids at pH 3.85. The acid adapted *S. typhimurium* also exhibited higher survival rates than their nonacid adapted counterparts during 5 hr of milk fermentation (pH 5.15 to 4.27 and 37°C). In mozzarella, cheddar and Swiss cheeses stored in anaerobic conditions at 5°C, acid adapted *S. typhimurium* survived for 54, 40 and 14 days, respectively, while their nonacid adapted counterparts survived for 28, 14 and 7 days, respectively. *Salmonella enteriditis, Salmonella heidelberg* and *Salmonella javiana* also displayed increased acid tolerance for lactic acid after acid adaptation. Leyer and Johnson (1993) also demonstrated that acid adapted *S. typhimurium* were more tolerant than their nonacid adapted counterparts when exposed to heat (50°C), NaCl (2.5 M), crystal violet (25 mg/L) or polymyxin B sulfate (10 mg/L).

Dickson and Kunduru (1995) inoculated sliced beef round with acid adapted (pH 5.0 and 37°C) or nonacid adapted strains of *Salmonella dublin, S. heidelberg* and two strain of *S. typhimurium.* The inoculated tissue was rinsed (10 s) in 1.5 or 3.0% lactic acid solutions at 23 or 55°C. The acid adapted strains for all species investigated were either more sensitive or equally sensitive to lactic acid when compared to their nonacid adapted counterparts. The decimal reduction times at 55°C ($D_{55°C}$ values) of acid and nonacid adapted strains of *S. typhimurium* and *S. dublin* were determined. The $D_{55°C}$ values were 7.86 and 13.44 min for acid adapted and nonacid adapted *S. typhimurium,* respectively, and 8.69 and 10.54 min for acid adapted and nonacid adapted *S. dublin,* respectively. The acid adapted cells exhibited lower D_{55C} values than nonacid adapted cells, indicating that acid adaptation of *Salmonella* spp. does not enhance the bacteria's ability to tolerate lactic acid or heat stress.

Antimicrobial activity of lactate salts

Sodium lactate inhibits the growth of *L. monocytogenes, C. botulinum, Clostridium sporogenes* and *P. fragi* (Maas et al., 1989; Harmayani et al., 1991; Shelef and Yang, 1991; Unda et al., 1991; Houtsma et al., 1994; Wederquist et al., 1994). Maas et al.

(1989) inoculated a comminuted turkey product with 10 strains of *C. botulinum*, held them at 27°C and evaluated their toxicity every day for 10 days. When sodium lactate was incorporated at 0, 3.0 and 3.5%, toxicity occurred at 3, 7 and 7 to 8 days, respectively. Houtsma et al. (1994) evaluated the effects of sodium lactate, sodium chloride and incubation temperature on the growth and toxin production of *C. botulinum*. At 15°C, 2 and 3% sodium lactate delayed growth and toxin production for 14 and > 49 days, respectively. At 20°C, 2.5% sodium lactate delayed growth and toxin production for 11 days. A combination of 2.1% sodium chloride and 2.5% sodium lactate inhibited growth for 15 days at 20°C. Sodium chloride (2.1%) alone allowed growth and toxin production within 5 days. Thus, sodium lactate reduces the growth and toxin production of *C. botulinum*.

Shelef and Yang (1991) showed that sodium lactate levels of greater than 5% delayed growth of 3 strains of *L. monocytogenes* in tryptic soy broth. They also inoculated sterile comminuted beef and chicken with *L. monocytogenes* and stored them at 35, 20 and 5°C. The addition of 4% sodium lactate suppressed growth in both beef and chicken. However, there was greater suppression in beef. Similarly, sodium lactate at 2 and 3% also suppressed *L. monocytogenes* growth in pork liver sausage stored at 5°C (Weaver and Shelef, 1993). Wederquist et al. (1994) inoculated sliced, vacuum packaged turkey bologna with *L. monocytogenes*. Bologna containing 2% sodium lactate exhibited 4 $\log_{10}$ CFU/g less than a cured control bologna after storage at 4°C for 40 and 90 days.

Unda et al. (1991) evaluated the growth and survival of *L. monocytogenes* cells and *C. sporogenes* spores in vacuum-packaged, nitrite-free, beef roasts prepared with brines containing selected antimicrobial agents. They observed that 2% sodium lactate inhibited the growth of anaerobic bacteria, *C. sporogenes* and *L. monocytogenes* in roasts that were temperature abused (24 hr @ 25°C) then stored at 2-4°C for 2 weeks. Sodium lactate inhibited the growth of *C. sporogenes* when roasts were stored at 2-4°C for 2 weeks or temperature abused (48 hr @ 25°C) then stored at 2-4°C for 2 weeks.

Miller and Acuff (1994) investigated the antimicrobial effects of sodium lactate on selected pathogens in cooked roast beef stored at 10°C for 28 days. Sodium lactate at 3 and 4% (raw, dry weight basis) suppressed the growth of surface inoculations of *S. typhimurium*, *L. monocytogenes* and *E. coli* O157:H7 when compared to 0 and 2% sodium lactate. *S. aureus* and *C. perfringens* did not grow on the beef roasts. Their numbers were not affected ($P < 0.05$) by the concentration of sodium lactate in the roasts.

Weaver and Shelef (1992) evaluated the antilisterial activity of sodium lactate, potassium lactate and calcium lactate in pork liver sausage stored at 5 or 20°C. All forms of lactate exhibited antilisterial activity. At 2 to 4%, potassium lactate and calcium lactate were more effective than sodium lactate at either storage temperature. It was also observed that the antilisterial activity of the lactates at 2 to 4% was enhanced by the presence of 2 to 4% sodium chloride.

Sodium lactate exhibits synergistic antimicrobial effects with other antimicrobial agents. Buncic et al. (1995) reported that the combination of sodium lactate (4%) and nisin (400 IU/ml) in BHI broth, resulted in an initial 1 $\log_{10}$ CFU/ml reduction in the population of *L. monocytogenes* and prevented regrowth for 14 days at 4°C. The combination of sodium lactate (4%), potassium sorbate (0.3%), nisin (400 IU/ml), sodium nitrite (0.0125%) and polyphosphate (0.5%) reduced the *L. monocytogenes* population by 3.5 $\log_{10}$ after 12 days at 4°C.

Kouassi and Shelef (1995) evaluated the effects of organic acid salts on the secretion of listeriolysin O (LLO) by *L. monocytogenes.* LLO is a sulfhydryl-activated protein, MW 55,000 to 60,000, produced by all virulent *L. monocytogenes* strains. There is the potential that compounds that stimulate or suppress LLO secretion may influence food safety. Sodium citrate, lactate and acetate enhanced LLO secretion during incubation at 35°C, which may suggest increased virulence of *L. monocytogenes*. Potassium sorbate suppressed LLO secretion. Combining sorbate, an LLO blocking agent, with lactate, an antilisterial agent, may prolong shelf-life and increase the safety of foods.

Mode of action

Doores (1993) indicated that the general mode of action for organic acids against microbial growth appears to be related to the maintenance of acid-base equilibrium, proton donation and the production of energy by the cells. Specific theories on lactate's mode of action include lowering the intercellular pH, lactate ions interfering with cellular functions, reducing water activity and iron chelation (Shelef, 1994).

In regard to reducing intercellular pH, lipophilic organic acid molecules in the protonated form (ie. undissociated) freely diffuse across cell membranes. If the extracellular pH is less than the intracellular pH the acid enters the cell and dissociates, releasing protons that acidify the cytoplasm. The cell attempts to maintain a constant internal pH by removing the protons. Since the cell is expending substantial energy to maintain a constant internal pH the growth rate is reduced. Interference with the proton gradient across the cell membrane further disrupts cell functions, such as amino acid transport. The acidification of the cytoplasm causes structural changes in proteins, nucleic acids and phospholipids and ultimately results in cell death (Shelef, 1994).

Ita and Hutkins (1991) studied changes in the intracellular pH of *L. monocytogenes* in the presence of lactic acid and other weak organic acids. The cells maintained a pH gradient of 1.0 to 1.5 pH units over a medium pH range of 3.5 to 6.5. At an external pH of 3.5 the intracellular pH remained at 5.0. These results suggest that the inhibition of *L. monocytogenes* is not directly caused by a decrease in intracellular pH, but rather by specific effects of undissociated acid species on metabolic or other physiological activities.

Maas et al. (1989) observed that lactate concentration rather than sodium concentration, ionic strength or brine content was the principal antibotulinal factor when sodium lactate and sodium chloride were incorporated into reinforced clostridial media. The specific mode of action was not known, however two possible mechanisms were proposed. First, the presence of high levels of lactate ion may inhibit a major anaerobic energy metabolism pathway that is essential for growth by shifting the pyruvate-reduction -to-lactate closer to its thermodynamic equilibrium. Second, high concentrations of extracellular lactate may inhibit lactate efflux from the cell, thus reducing ATP generation from proton transfer across the cell membrane.

There is some evidence that the antimicrobial activity of lactate salts may be associated with affects on water activity (Shelef, 1994). Chen and Shelef (1992) evaluated the relationship between a_w, lactate salts and the growth of *L. monocytogenes* in a model meat system consisting of strained cooked beef ranging in moisture content from 25 to 85%. Lactate (4%) depressed meat a_w. As the moisture content decreased the differences in the a_w between the control and the 4% lactate progressively increased from 0.003 (85% moisture) to 0.046 (25% moisture). This trend may have enhanced the inhibitory activity of lactate as moisture decreased. However, the depression of a_w alone does not explain the observed inhibition by lactate. Listeriostatic effects by 4% lactate were observed at 55% moisture and a a_w of 0.964. Listeriostatic effects without lactate occurred at 25% moisture and a a_w of 0.932. Listerial growth without lactate was evident at 35% moisture and a a_w of 0.964. This was comparable to the a_w of 4% lactate and 55% moisture and supports the observation that the concentration of lactate ions is a factor for antimicrobial activity (Maas et al., 1989).

Shelef (1994) proposed that lactate's antimicrobial activity on *L. monocytogenes* and *C. botulinum* may be related to lactate's ability to chelate iron. This proposal was supported by the observation that lactate stabilizes fats and oils by the probable mechanism of chelating trace amounts of iron. Weaver and Shelef (1993) also observed that the antilisterial activity of lactates in pork liver sausage is enhanced by heat treatment. Sodium lactate in pork liver sausage showed superior antilisterial activity in sausage that was sterilized (15 min at 121°C) when compared to a heat processed sausage (water bath, 70°C meat internal temperature). The high temperature of the sterilized product may have contributed to increased chelation of polyvalent cations essential for the growth of *Listeria*.

Applications of lactic acid on/in meat

Lactic acid has been applied to beef, pork and chicken carcasses as a method of microbial decontamination. Hamby et al. (1987) reported that intermittently spraying (two 30 s sprays/hr for 12 hr) 1% lactic acid on sides of beef resulted in 0.8 to 2.4 $\log_{10}$ CFU/cm^2 reductions in aerobic growth on the inside round, strip loin, boneless ribs, clod or neck than nonsprayed control sides of beef after 48 hr of storage (2°C). After fabrication and vacuum packaged storage (28 days at 2°C) the inside round, strip loin, boneless rib and clod from acid treated sides of beef exhibited 0.7 to 3.3 $\log_{10}$ CFU/cm^2 less aerobic growth than subprimal cuts from control sides of beef.

Prasai et al. (1991) evaluated the effects of a hot (55°C) 1% lactic acid spray applied to beef carcasses after dehiding, after evisceration and at both locations during slaughter on aerobic microbial growth. Immediately post-slaughter, acid treated carcasses exhibited 0.9 to 2.3 $\log_{10}$ CFU/cm^2 less growth than control nonacid treated carcasses. Among the acid treatments, carcasses that were sprayed once (after evisceration) and twice (after dehiding and after evisceration) exhibited lower ($P < 0.05$) aerobic plate counts than carcasses sprayed after dehiding. Similar trends were observed for microbial growth after 72 hr at 1°C. However, in vacuum packaged beef loins (3 and 14 days post-fabrication) no differences in aerobic growth were observed as a result of lactic acid spray. When a 1% hot lactic acid spray was applied to carcasses after dehiding and after evisceration in commercial slaughter facilities immediate reductions in aerobic microbial growth were observed. Levels of reduction were 1.3 $\log_{10}$ CFU/cm^2 at a cow-slaughter facility, 1.5

$\log_{10}$ CFU/cm^2 at a large slaughter facility and 1.9 $\log_{10}$ CFU/cm^2 at a small slaughter facility.

Prasai et al. (1992) applied a hot (55°C) 1% lactic acid spray to pork carcasses immediately after dehairing, immediately after evisceration and at both locations during slaughter, and evaluated the aerobic growth on the surface of the carcasses during chilling and after fabrication. Acid treatment did not significantly ($P > 0.05$) affect aerobic growth on the pork carcasses or on vacuum packaged loins.

Sawaya et al. (1995) dipped whole chicken carcasses for 1 min in a 10% (wt/vol) lactic acid/sodium lactate buffer (0.902 mol/L lactic acid and 0.2082 mol/L sodium lactate) and packaged the carcasses in either conventional aerobic packaging or under modified atmosphere (70% CO_2, 5% O_2 and 25% N_2). Pretreatment with lactic acid/sodium lactate buffer of conventionally packaged carcasses resulted in a shelf-life of 13 days at 4°C and 10 days at 7°C. The shelf-life was increased by 6-7 days at 4°C and 5-6 days at 7°C. The pretreated, modified atmosphere packaged (MAP) carcasses exhibited a shelf-life of > 36 days at 4°C and 35 days at 7°C. The shelf-life of the pretreated carcasses was longer than the untreated MAP carcasses by > 14 days when stored at 4°C and by 22 days when stored at 7°C.

Abugroun et al. (1993) utilized a 1.25% lactic acid dip (pH 2.18) in a system to extend the shelf-life of unrefrigerated, prerigor cooked beef. They reported that after 14 days of storage (22°C) lactic acid dipped samples cooked in 100°C water for 40 min exhibited 1.1 $\log_{10}$ CFU/g less aerobic growth than similar samples dipped in water.

Ogden et al. (1995) used lactic acid (0.09, 0.19 or 0.29 mol/L) to preserve ground pork for 12 days at 4°C. The pH of the samples were 5.4, 4.6, 4.25 and 4.0 for the untreated control, 0.09, 0.19 and 0.29 mmol/L lactic acid treatments, respectively. After 12 days, lactic acid treatments exhibited 3 to 4.5 $\log_{10}$ CFU/g less aerobic growth and 2 to 6 $\log_{10}$ CFU/g less *Pseudomonas* growth than the control. Higher concentrations of lactic acid resulted in lower levels of microbial growth. During storage, when lactic acid concentration was increased from 0.09 to 0.29 mmol/L, the hexanal, 2-pentyl furan and 2-ethyl furan production increased from 2 to 6 times that of the water treated control. The increase in hexanal, 2-pentyl furan and 2-ethyl furan production indicated that increasing the lactic acid concentration resulted in lipid oxidation. After 12 days, the control exhibited 2 to 6 times more dimethyl disulfide production than treatments with lactic acid. Dimethyl disulfide imparts an undesirable "rotten egg" odor to meat. It is reported to be produced by *Pseudomonas* spp. The trends of dimethyl disulfide production followed the growth trends of *Pseudomonas* spp. in ground pork. Lactic acid treatments resulted in lower L-values indicating a browner meat sample which was likely the result of metmyoglobin formation. Treatment with 0.09 mmol/L lactic acid exhibited minimal changes in the color of the ground pork. Lactic acid (0.09 mmol/L) was considered to be an acceptable method of extending the shelf-life of ground pork.

Applications of lactate salts in meat

The lactate salts of sodium, potassium and calcium have been incorporated into roast beef (Papadopoulos et al., 1991a, 1991b; Unda et al., 1991; Miller and Acuff, 1994), turkey bologna (Wederquist et al., 1994), pork liver sausage (Weaver and Shelef, 1993), ground pork (O'Connor et al., 1993; Nnanna et al., 1994; Brewer et al. 1995), pork sausage (Brewer et al., 1991; Lamkey et al., 1991; Bradford et al., 1993a, 1993b), ground beef patties (Egbert et al., 1992) and catfish fillets (Williams et al., 1995). In addition to the previously discussed antimicrobial activities, researchers reported that lactate salts typically did not exhibit negative effects on the color or flavor of the products to which they were applied. In several cases lactate salts were reported to enhance flavor, protect color and inhibit lipid oxidation.

Papadopoulos et al. (1991a, 1991b) evaluated the effects of sodium lactate on roast beef. The addition of 3% sodium lactate reduced microbial growth. Over an 84 day storage period, the microbial distribution shifted from primarily *Micrococcus* and coagulase negative *Staphylococcus* spp. to hetero- and homo-fermentative *Lactobacillus* spp. Sodium lactate at > 3.0% minimized pH decline during storage (Papadopoulos et al., 1991a). A trained flavor profile panel revealed that 3% sodium lactate enhanced beef/brothy, metallic/sweet aromatic and serumy/bloody flavors and saltiness in roast beef. Sodium lactate also minimized painty, cardboard and fishy flavors. Consumer evaluation indicated that sodium lactate intensified sourness, saltiness, beef flavor and overall flavor intensity. Consumers also preferred roast beef with sodium lactate for the overall like/dislike of the product, overall flavor, beef flavor and texture (Papadopoulos et al., 1991b). Similarly, the addition of 2 or 3% sodium lactate to fresh pork sausage delayed microbial deterioration, pH decline and development of sour and off-flavors. It also protected red color and enhanced pork and salty flavors in fresh pork sausage (Brewer et al., 1991; Lamkey et al., 1991).

The effects of combinations of sodium lactate and sodium chloride on the sensory characteristics, microbial growth and color of fresh ground pork have been investigated. O'Connor et al. (1993) reported that the addition of 1.5 and 3.0% sodium lactate to ground pork (20% fat) enhanced its juiciness, salt intensity and pork-flavor. When compared to controls, sodium lactate (3%) reduced ($P < 0.05$) aerobic growth and extended the shelf-life, at 4°C by about 12 days, based on a spoilage level of 10^6 CFU/g. Similarly, Brewer et al. (1995) evaluated the effects of combinations of sodium lactate and sodium chloride on the color and microbial growth of aerobically packaged ground pork (15% fat). Redness was best preserved by 2.0 or 3.0% sodium lactate. Samples containing 3.0% sodium lactate exhibited the lowest aerobic growth regardless of salt level.

Sodium lactate has also been applied at 0, 1 and 2% to catfish fillets that were stored under simulated retail conditions (Williams et al., 1995). The fillets with 2% sodium lactate exhibited lower ($P < 0.05$) aerobic plate counts and thiobarbituric acid (TBA) values than fillets treated with 0% sodium lactate. The color, texture, flavor and odor of the cooked catfish fillets were not significantly ($P > 0.05$) affected by sodium lactate. However, approximately 10% of the panelists consistently identified an objectionable "metallic", "sodium" or "chemical" aftertaste in the fillets with 2% sodium lactate.

The effects of potassium lactate (0, 1, 2 and 3%) on the properties of low-fat, carrageenan-based beef patties were evaluated by Egbert et al. (1992). Potassium lactate (2 and 3%) reduced bacterial growth with no deleterious effects on visual or organoleptic properties of the low-fat beef patties.

Bradford et al. (1993a and 1993b) evaluated the effects of using potassium lactate in fresh pork sausage. In typical pork sausage patties (40% fat), and low-fat (8% fat) patties, less than 2% potassium lactate did not affect the percent discoloration or lean color, sensory properties of the sausage, TBA reactive substance (TBA-RS) values or 'L', 'a' and 'b' values during refrigerated aerobic storage. However, in typical patties, 2% potassium lactate reduced ($P < 0.05$) microbial numbers during refrigerated storage when compared to typical fresh pork sausage without potassium lactate (Bradford et al., 1993a). Similarly, Bradford et al. (1993b) utilized 2% potassium lactate in typical pork sausage (25% fat) and low-fat (8% fat) sausage chubs. Typical sausage with potassium lactate had lower ($P < 0.05$) psychrotrophic counts than sausage without sodium lactate. Potassium lactate delayed ($P < 0.05$) pH decline in typical sausage but had no effects on low-fat sausage ($P > 0.05$).

Nnanna et al. (1994) evaluated the antioxidant activity of sodium lactate in meat and a model system. The meat system consisted of raw pork (19% fat, 63.9% moisture) mixed with BHT (0.1 g/kg fat) or with sodium lactate (10 or 20 g/kg meat) stored in the dark at 0 or 5°C. After 10 days at 0°C, the TBA-RS values of the BHT and sodium lactate treatments were not significantly different ($P < 0.05$) than the control. After 14 days, the TBA-RS values for all treatments were lower ($P < 0.05$) than the control, and the 10g/kg sodium lactate treatment was higher ($P < 0.05$) than the 20 g/kg sodium lactate and BHT treatments which were not significantly ($P > 0.05$) different from each other. After 7 days at 5°C, the TBA-RS values for all treatments were lower than the control and the 10 g/kg sodium lactate treatment was higher ($P < 0.05$) than the 20 g/kg sodium lactate and BHT treatments, which were not significantly different from each other. After 10 days, there were no significant ($P > 0.05$) differences between any of the treatments stored at 5°C. Thus, sodium lactate exhibited antioxidant effects that were dependent upon lactate concentration and temperature.

The model system consisted of methyl linoleate (2.5 mmol/L) dispersed in phosphate buffer (25 mmol/L) with oxidation being initiated by ferric chloride (150 μmol/L) and ascorbate (100 μmol/L) in a total reaction volume of 5 ml (Nnanna et al., 1994). The system evaluated the antioxidant activity of sodium lactate (5 or 10 g/L) with reference to BHT (0.1 g/L) and BHA (0.1 g/L). The tests were conducted in a shaker water-bath at 25±1°C under subdued light. The extent of oxidation was monitored by measuring peroxide value, conjugated diene and TBA-RS at various time intervals. After 24 hr, the peroxide values for the control, 5 and 10 g/L sodium lactate treatments were 40, 20 and 50 meq/kg of linoleic acid (LA) higher than the BHA and BHT treatments, respectively. After 48 hr, the conjugated diene values for the BHA and BHT treatments were 50, 42 and 15 units lower than the control, 10 g/L and 5 g/L sodium lactate treatments, respectively. Sodium lactate was more effective in controlling both peroxide value and conjugated diene formation when the concentration was 5 g/L. After 72 to 144 hr the BHA, BHT and sodium lactate treatments were comparable for TBA-RS values with values ranging from 20 to 30 µmol/kg LA. The control exhibited TBA-RS values of 75 µmol/kg LA at 72 hr and a 225 µmol/kg LA peak at 120 hr. The effectiveness of sodium lactate in the tested parameters was TBA-RS > conjugated diene formation > peroxide value. This indicates that in the ferric iron and ascorbate system sodium lactate controls the formation of secondary products, such as ketones and aldehydes. Calcium lactate was shown to have similar antioxidant characteristics as sodium lactate. The proposed mechanism for antioxidant activity of lactate is an interaction of lactate with a peroxy radical to form a lactyl radical, which interacts with ferric iron to form pyruvate and ferrous iron.

Gluconic Acid and Glucono-Delta-Lactone

There is limited published information on the use of gluconic acid or glucono-delta-lactone (GDL) in foods. This review addresses gluconic acid and GDL's physical and chemical characteristics, antimicrobial activity, mode of action and applications in meat.

Chemical characteristics

Glucono-delta-lactone is the cyclic 1,5-intramolecular ester of D-gluconic acid. It is prepared by the direct crystallization of aqueous gluconic acid (USFDA, HHS, 1995). GDL has a solubility in water of 59 g GDL/100 ml water at 25°C. It is readily hydrolysed to gluconic acid by water. A 1% aqueous solution of GDL has an initial pH of 3.6 which changes to pH 2.5 within 2 hr (Merck & Co., Inc., 1989). GDL exhibits an ionization constant of 1.99×10^4 and a pKa of 3.7. GDL has a sweet taste while gluconic acid has a mild acid taste. The acidic nature and mild flavor make GDL and gluconic acid ideal acidifiers for delicately flavored food (McCarthy et al.,1991).

GDL has GRAS status as a direct human food ingredient for the purposes of curing and pickling agent, leavening agent, pH control agent and sequestrant (USFDA, HHS, 1995). In the U.S., GDL is allowed in sausages at a level of 0.5%, except in Genoa salami where 1% GDL is permitted.

Antimicrobial activity

Gluconic acid and GDL exhibit a range of antimicrobial activity. They increase the thermal sensitivity of *Bacillus* spp. (Oscroft et al., 1990; Fernandez et al., 1994) and *C. sporogenes* (Silla-Santos et al., 1992; Ocio et al., 1994) spores. They also inhibit the growth of *L. monocytogenes* (El-Shenaway and Marth, 1990), *S. aureus* (Metaxopoulos et al., 1981); lactic acid and psychrotrophic bacteria (Madden, 1989; Zepeda et al., 1994). Gluconic acid and GDL have also been reported to exhibit synergistic antimicrobial effects with nisin (Oscroft et al., 1990), lactic acid (Zepeda et al., 1994) and sodium lactate (Qvist et al., 1994).

Concentrations of 0.05 and 0.5% (w/v) GDL are effective in inhibiting the outgrowth of *Bacillus* spores heat treated to 95°C and incubated in trypticase soy broth. However, when spores were heat shocked at 65°C for 1 hr, only 0.5% GDL had inhibitory effects on spore outgrowth at 20 and 30°C (Oscroft et al., 1990). Acidifying mushroom extract with GDL to pH values below 6.22, diminishes the thermal resistance of *Bacillus stearothermophilus* ATCC 12980 spores (Fernandez et al., 1994). Similarly, acidification with GDL reduces the thermal resistance of *C. sporogenes* (PA 3679) spores in white asparagus puree (Silla-Santos et al., 1992) and mushroom extract (Ocio et al., 1994).

Oscroft et al. (1990) observed the effects of incubation temperature, pH, nisin and organic acids on the growth of thermally stressed *Bacillus* spores in trypticase soy broth. Both GDL (0.05% and 0.5%) and nisin (125 to 1000 IU/ml) delayed the outgrowth of thermally stressed (65°C, 1 hr) *Bacillus* spores. Synergistic effects occurred between GDL and nisin at pH conditions of 4.8 to 6.0. Spore outgrowth was completely inhibited by the combination of 0.5% GDL, 125 to 1000 IU/ml nisin, 4.2 to 5.8 pH and 15 min of thermal stress at 95°C.

El-Shenaway and Marth (1990) evaluated the growth of *L. monocytogenes* in tryptose broth and reconstituted milk in the presence of gluconic acid or glucono-delta-lactone and incubated at 13°C. The use of gluconic acid delayed onset of growth when concentrations were 0.25% (pH 5.29) or greater in both the broth and milk. As gluconic acid concentration increased, pH decreased. The addition of GDL to tryptose broth resulted in delayed onset of growth at 0.2% and inhibition at 0.4%. In milk, 0.5 and 1% GDL delayed onset of growth, while 1.5% and greater concentrations inhibited growth. Glucono-delta-lactone gradually acidified milk and tryptose broth within 24 hr. The use of 0.5% GDL reduced pH from 6.1 to 5.3 in milk and from 6.4 to 4.5 in broth. Similarly, 1% GDL reduced pH from 6.0 to 4.9 in milk and from 5.5 to 3.7 in broth.

Metaxopoulos et al. (1981) utilized GDL at 0.4 and 1% to reduce the initial pH of an Italian-style dry sausage from an initial pH of 6.1 to 5.5 and 4.8, respectively. When used alone or in conjunction with lactic acid bacteria the addition of GDL resulted in a

2-3 $\log_{10}$ CFU/g reduction in *S. aureus* strain S-6 during fermentation and 17 days of ripening.

Madden (1989) reported that GDL at 0.5% (w/v) in pork liver paté inhibited the growth of lactic acid bacteria. It also reduced the occurrence of greening or souring. However, GDL allowed yeasts to proliferate.

Zepeda et al. (1994) evaluated the potential for using gluconic acid as a fresh beef decontaminant. When semimembranosus samples were subjected to a 2 min dip in 1.5% or 3.0% gluconic acid there was a 1 $\log_{10}$ reduction in lactic acid bacteria count. There was also a trend of reduced psychrotrophic counts as gluconic acid concentration increased. Combining 1.5% lactic acid with 1.5 or 3.0% gluconic acid enhanced the antimicrobial activity. During 28 days of vacuum packaged storage (1°C), 1.5 and 3.0% gluconic acid treatment exhibited psychrotrophic plate counts and lactic acid bacteria counts that were 0.5 to 1.5 $\log_{10}$ CFU/cm^2 lower than control. Combinations of lactic acid and gluconic acid resulted in a maximum of 2.5 $\log_{10}$ CFU/cm^2 less than the control. Combinations of lactic acid and gluconic acid were more effective at inhibiting the growth of lactic acid and psychrotrophic bacteria than either acid alone. The enhanced antimicrobial activity for gluconic acid may have been the result of increased acidity on the semimembranosus samples. The pH values for acid treatments and treated semimembranosus pHs at day 0 were 5.62 for the water control, 5.56 for 1.5% gluconic acid, 5.46 for 3.0% gluconic acid, 5.41 for 1.5% lactic acid, 5.39 for 1.5% gluconic acid and 1.5% lactic acid and 5.40 for 3.0% gluconic acid and 1.5% lactic acid. Within the gluconic acid treatments the theory of 'reduced pH results in increased antimicrobial activity' appears to be true. However, when lactic acid is combined with gluconic acid the pHs of the meat sample are similar indicating that there may be some form of synergistic interaction between the acids that is not reflected in the pH.

Qvist et al. (1994) produced bologna style sausage with four formulations: a) standard commercial formulation (control), b) 2% sodium lactate with no GDL, c) 2% sodium lactate and 0.25% GDL and d) 2% sodium lactate and 0.5% GDL. Sliced sausage was inoculated with a mixture of *L. monocytogenes* serotype 1 and 4 at approximately 100 CFU/g. The product was vacuum packaged and stored at 5 or 10°C for 35 days. Rapid growth occurred in the control stored at 5 and 10°C and in the 2% sodium lactate treatment stored at 10°C. The 2% sodium lactate treatment stored at 5°C suppressed growth for 28 days. Addition of GDL at 0.25 and 0.5% inhibited growth for 35 days at both storage temperatures.

El-Khateib and El-Rahman (1987) reported that 0.3% GDL, 2.5% nitrate curing salt and *Lactobacillus plantarum* (10^7/g) in Egyptian fresh sausage stored at 25°C resulted in a 1 $\log_{10}$ CFU/g reduction in *S. typhimurium* counts after 2 days of storage. A control sausage and sausage containing *L. plantarum* (10^7/g) exhibited 2 and 1 $\log_{10}$ CFU/g of *S. typhimurium* growth, respectively, after storage under the same conditions.

Mode of action

As previously discussed Doores (1993) reviewed the organic acids indicating that their general mode of antimicrobial action appeared to be related to the acid-base equilibrium, proton donation and the production of energy by the cells. This particular review did not include gluconic acid or glucono-delta-lactone, however since gluconic acid is an organic acid it should stand to reason that gluconic acid acts in a similar manner as other organic acids. Therefore, as described by Shelef (1994), at low pH, undissociated gluconic acid diffuses across the cell membrane where it dissociates, acidifying the cytoplasm. In an attempt to maintain homeostasis, the cell expends substantial energy and reduces its growth rate. If the cell is unable to maintain homeostasis the cytoplasm will acidify causing structural changes in proteins, nucleic acids and phospholipids which will lead to death.

Applications of gluconic acid or glucono-delta-lactone in meat

In addition to its antimicrobial applications in nonmeat foods and fermented meat products, GDL has been shown to be a useful acidulant for cured meat production. In the presence of water or when heated it hydrolyses to gluconic acid, thus lowering the pH of the meat product and accelerating the development of cured color. The addition of 1% GDL typically reduces the pH of a meat product by 1 pH unit, while 0.5% will reduce pH by approximately 0.5-0.6 of a pH unit (Haymon, 1981).

Acton and Dick (1977) incorporated 0.41% GDL into fermented sausages. GDL immediately reduced meat pH from 6.0 to 5.4. During fermentation and heat processing the additional acidity from GDL promoted greater ($P < 0.05$) conversion of the total heme pigments to nitric oxide heme pigments. However, during dehydration sausages with GDL lost pigments at the same rate as controls.

Pate et al. (1971) utilized GDL (0, 30 or 60 g/L of brine) in cured hams to evaluate its effects on cured color and color stability. The addition of GDL did not significantly affect the nitric oxide pigment or total pigment formation and stability of the pigments after aging for 1 or 14 days or exposure to light (200 ft-c) for 0, 30, 60, 90, 120,150, 180 and 1440 min. However, as GDL concentration increased the moisture content of the hams decreased by almost 4%.

Ethylenediaminetetraacetic Acid

Shelef and Seiter (1993) reviewed the physical and chemical properties, antimicrobial activity, mode of action, applications, regulatory status, toxicology and assay of ethylenediaminetetraacetic acid (EDTA) in foods. This review will focus on EDTA's physical and chemical properties, antimicrobial activity and mode of action.

Physical and chemical properties

Ethylenediaminetetraacetic acid (EDTA) is a metal chelating agent with four carboxyl (-COOH) groups and two amine groups (-NR_2). The nitrogen atoms and free electron pairs of two of the anionic oxygen atoms of two carboxyl groups initially interact with a cationic metal (e.g. Ca^{+2}, Fe^{+2}). The metal-EDTA complex has a spatial configuration that allows additional coordination of the metal with the free electron pairs of the anionic oxygen atoms of the two remaining carboxyl groups. This results in an extremely stable complex utilizing all six electron donor groups (Lindsay, 1985).

The strong chelating abilities of EDTA help it to function as both an antioxidant and antimicrobial agent. Lipid oxidation is often stimulated by transition metals (e.g. cobalt, copper, iron, manganese and nickel) possessing two or more valency states with suitable oxidation-reduction potential between them (Nawar, 1985). The antioxidant activity of EDTA involves binding metal cations and preventing their interaction with lipids, thus preventing oxidation.

The USFDA allows a maximum of 0.0036% disodium EDTA in cooked sausages. The maximum levels allowed in any food product is 0.05% in canned strawberry pie (USFDA, HHS, 1995).

Antimicrobial activity

Winarno et al. (1971) reported that EDTA (2.5 to 10 mM) was inhibitory to the germination of and outgrowth from spores of *C. botulinum* type A in peptone broth. The inhibitory action of EDTA was enhanced as the broth's pH was increased from 6.5 to 8.1. The inhibitory action was completely erased when equimolar concentrations of $CaCl_2$ or $MgCl_2$ were present. When EDTA was incorporated into fish homogenate 5.0 mM was required to inhibit the germination of and outgrowth from *C. botulinum* spores.

Okereke et al. (1990b) utilized $CaNa_2EDTA$ (200 ppm, 0.02%) in combination with acid blanching to prepare canned mushrooms from mushrooms inoculated with 3.2 x 10^5 spores/g of proteolytic *C. botulinum* type A and B and noninoculated mushrooms. The canned mushrooms were heated to the equivalent of 0.0, 0.5, 2.0 or 4.0 min at 121°C (F_o). When compared to the standard canning method, acid blanching at F_o of 0.5, 2.0 and 4.0 reduced percent spoilage of the inoculated mushrooms from 91.4 to 51.6, 17.6 to 6.3 and 12.1 to 2.9, respectively. No botulinal toxicity occurred except at F_o 0.5, indicating that spoilage was due to other heat resistant endogenous mesophilic spores. In noninoculated mushrooms, acid blanching at F_o of 0.5 and 2.0 reduced percent spoilage from 44.4 to 31.6 and 15.8 to 0.0, respectively. At F_o 4.0 no spoilage occurred with either method. The acid blanch with EDTA system was also applied in a commercial cannery study in which the thermal process time was reduced by half.

The effects of EDTA, pH and divalent cations on the germination and outgrowth from spores of toxigenic *Bacillus cereus* strain USDA 201 have also been investigated. Bulgarelli and Shelef (1985) incubated (35°C, 48 hr) 10^4 heat activated *B. cereus* spores/ml in BHI broth containing 0 to 1000 ppm (0.1%) Na_2EDTA. EDTA did not affect the percent of spore germination. EDTA at 0.03 to 0.1% inhibited growth. Over a pH range of 5 to 9 the highest spore resistance to 0.05% (1.49 mM) EDTA was observed at pH 7. The addition of 1.00 and 1.49 mM of Fe, Zn, Ca or Mg interfered with EDTA's inhibitory action.

Levin (1967) reported that in half strength nutrient broth (pH 7.0) Na_4EDTA (0.005%) markedly inhibited the growth of *Pseudomonas putrefaciens*, *P. fragi* and *Pseudomonas flourescens*. However, *Achromobacter lipolyticum* was insensitive to EDTA. Dipping haddock fillets in a 1.0% Na_4EDTA solution for 1 min failed to inhibit the growth of bacteria during refrigerated storage (3°C) when compared to untreated control fillets. However, based on odor and taste evaluations the dip extended refrigerated storage from 5 to 9-10 days.

Robach and Stateler (1980) reported that 50 ppm (0.005%) EDTA did not affect the growth of *S. aureus* S-6 or 12600 in trypticase soy broth (pH 6.0) at 37°C. Potassium sorbate's (0.1 or 0.2%) inhibitory activity toward both *S. aureus* strains was enhanced in the presence of 0.005% EDTA. Kraniak and Shelef (1988) observed that EDTA or its sodium or potassium salts (0.8-1.7 mM, ~0.025-0.05%) inhibited the growth of *S. aureus* strain 196E in BHI broth. The addition of equal concentrations of Fe, Zn or Ca to EDTA containing broth eliminated inhibition. The addition of Mg required an EDTA:Mg molar ratio of 1:7 to eliminate EDTA's inhibitory action.

Moustafa et al. (1969) reported that in lactose-yeast extract broth (13°C) *Pseudomonas fragi* grew to a lower maximum volume when EDTA was present at 0.75 mg/ml. When lysozyme (0.2 mg/ml) was added to EDTA (0.75 mg/ml) the maximum population of *P. fragi* was reduced by 2.5 $\log_{10}$ CFU/ml when compared to EDTA alone. In lactose-yeast extract (13°C), the combination of 1.0 mg/ml EDTA and 2 mg/ml lysozyme prevented the growth of *P. fragi*. The combination 0.75 mg/ml EDTA and 0.2 mg/ml did not effect the growth of *P. fragi* when the growth media was skim milk, half-and-half or when the lactose-yeast extract broth contained skim milk (2%), milk ash (0.12%) or magnesium sulfate (0.01 M). The loss of antimicrobial activity was associated with the presence of metal ions in each type of growth media.

Payne et al. (1994) evaluated the effects of combinations of disodium EDTA and lysozyme on the growth of *P. flourescens*, *S. typhimurium*, *L. monocytogenes* and *E. coli* O157:H7 in brain heart infusion broth (18 hr at 25°C for *P. flourescens* or 36°C for other organisms). Lysozyme and EDTA combinations did not affect ($P > 0.05$) the growth of *P. flourescens* or *S. typhimurium*. However, *E. coli* O157:H7's growth was reduced by 4 $\log_{10}$ cycles in the presence of 1.0 to 2.5 mg/ml EDTA. Lysozyme did not change EDTA's effects on the growth of *E. coli* O157:H7. When used alone neither EDTA nor lysozyme inhibited the growth of *L. monocytogenes*. However, combinations of 2.5 mg/ml EDTA and 100 μg/ml lysozyme, 2.0 mg/ml EDTA and 200 μg/ml lysozyme and

2.5 mg/ml EDTA and 200 μg/ml lysozyme reduced growth by 4.7, 4.35 and 5.52 $\log_{10}$ cycle, respectively, when compared to a no additive control.

Teotia and Miller (1975) inoculated turkey drumsticks with 500 ml of 0.1% peptone water containing 5-8 x 10^5 CFU/ml of *S. senftenberg* 775W. Dipping the contaminated drumsticks in a 0.5% solution of EDTA (21°C) for 180 min failed to destroy *S. senftenberg*. However, dipping in solutions of 0.1% lysozyme, 0.1% lysozyme and 0.5% EDTA or 0.5% lysozyme and 1.0% EDTA under similar conditions was bactericidal.

In trypticase soy broth, 0.5% EDTA inhibited the growth of *S. typhimurium* and *S. heidelberg* incubated at 37°C for 10 hr (Samuelson et al., 1985). Stevens et al. (1991) observed that the combination of EDTA (20 mM) and nisin (50 μg/ml) in cell buffer (Tris-HCl) incubated at 37°C resulted in a 3.2 to 6.9 $\log_{10}$ cycle reduction in various species of *Salmonella* and other Gram-negative bacteria. However, neither EDTA nor nisin produced significant inhibition when used alone.

Golden et al. (1995) evaluated the inactivation time of *L. monocytogenes* in BHI broth (28°C) treated with EDTA (0.16%) or a combination of EDTA, ascorbic acid (0.04%) and sodium acetate (1.8%) or sodium propionate (1.8%). When EDTA was used alone it did not affect the inactivation time of *L. monocytogenes*. However, when used in combination with ascorbic acid and sodium acetate or ascorbic acid and sodium propionate there was approximately a 5% decrease in the inactivation time when compared to the organic acids alone.

Mode of action

For Gram-negative bacteria EDTA's antimicrobial activity is thought to be the result of it binding divalent metal cations found in the cell walls of organisms. This increases cell permeability and facilitates action of antimicrobial agents or degradative enzymes, releases lipopolysaccharides from the outer membrane and may cause lysis. In Gram-positive organisms the mechanism of inhibition is thought to be chelation of cell wall cations in a similar manner as in Gram-negative organisms or by chelating nutrients essential for bacterial growth (Kraniak and Shelef, 1988).

Ethylenediaminetetraacetic acid (500 μg/g) has been utilized in combination with sodium nitrite (156 μg/g) and sodium isoascorbate (200 μg/g) to reduce the growth of *C. botulinum* (47-96 spores/g after cooking) in canned cured pork hams and pork hearts (Tompkin et al., 1978 and 1979). In canned pork hearts, 100% of the cans containing meat treated with nitrite alone swelled after 10 days of storage (27°C), while the combination of EDTA and nitrite exhibited ~4% swelled cans at 40 days and 100% of the cans swelled by 60 days. The proposed mechanism for EDTA's synergistic activity was that EDTA retarded the repair of nitrite injured cells by binding an essential cation (Tompkin et al., 1978). Tompkin et al. (1979) prepared canned cured hams with increasing levels of added iron (0 to 40 μg/g) or EDTA (50 to 400 μg/g) and inoculated them with *C. botulinum*. As the level of added iron increased, the cans swelled over a shorter period of time. When the EDTA concentration was increased more time was required for the cans to swell, indicating that iron may be the cation that EDTA binds to inhibit the repair and growth of nitrite injured *C. botulinum*.

Okereke et al. (1990a) investigated the mechanism by which the acid blanch - EDTA process inhibit the outgrowth of *C. sporogenes* PA 3679 spores. Inhibition appeared to be the result of heat at reduced pH destroying the spores natural germination system following the collapse of the cortex peptidoglycan layers, possible hydration of the core and concomitant increase in the core volume. $CaNa_2EDTA$ had no apparent effect on the spores during heat processing, however it may have sequestered the metal ions that were essential for the repair and germination of partially damaged spores.

Important Foodborne Pathogens

Escherichia coli O157:H7

Diarrhegenic *E. coli* have been thoroughly reviewed. Levine (1987) discussed five types of *E. coli* that cause diarrhea, their clinical patterns, epidemiology, pathogenesis and O:H serotypes. Doyle and Padhye (1989) reviewed the survival, growth, pathogenesis and epidemiology of *E. coli*. Doyle (1991) discussed *E. coli* O157:H7 and its significance in foods. Farrag and Marth (1992) reviewed the history, growth requirements, disease characteristics, outbreaks, occurrence in foods and reservoirs of *E. coli* O157:H7 and discussed the potential for controlling it in milk products with the lactoperoxidase system. Several reviews occurred in response to the 1993 *E. coli* O157:H7 outbreak in the Western United States. Reed (1994a) briefly discussed the sub-groups of diarrheal *E. coli* implicated in foodborne illness, sources of food contamination and measures for controlling food contamination. Tarr (1994) reviewed the progression and cause of the 1994 *E. coli* O157:H7 outbreak in the Western U.S. Neill (1994) traced the *E. coli*

O157:H7 cases and outbreaks from 1982 to 1993 and the acquisition of knowledge about the organism. Lior (1994) reviewed *E. coli* O157:H7 and verotoxigenic *E. coli*, their vectors of infection and serotypes that have been isolated in Canada. Duncan and Hackney (1994) discussed *E. coli* O157:H7's epidemiology, historical incidence and relationship to the dairy industry. Madden (1994) discussed various *E. coli* O157:H7 outbreaks and foods that have been or can be potential vectors for foodborne illness. McNamara (1994) also discussed several outbreaks and FSIS, Microbiology Division's surveillance and monitoring programs for raw beef and poultry. Rowbury (1995) assessed the environmental factors influencing the acid tolerance and sensitivity in *E. coli*. This review will focus on the growth requirements, disease characteristics, epidemiology and control of foodborne *E. coli* O157:H7.

Escherichia coli is a Gram-negative, facultative anaerobe in the family *Enterobacteriaciae*. It is capable of using simple sugars and minimal basal medium for growth. Standard biochemical tests for identifying *E. coli* include indole production, usually positive; methyl red, positive; Voges Proskauer, negative; and citrate, negative. Cells are short straight rods either motile with peritrichous flagella or nonmotile. Temperature range of growth is 5 to 45°C with 37°C being optimum. Minimum water activity is 0.94. Average pH range is 5.0 to 8.0. Strains are sensitive to heat, with a D_{60} of 1 min and irradiation with a D_{10} of 9 Krad (Doyle and Padhye, 1989).

Generally, *E. coli* strains that inhabit human intestines are harmless. However, there are pathogenic strains that cause distinct syndromes of diarrheal disease. The four main categories of disease include a) enteropathogenic *E. coli* (EPEC), b) enteroinvasive

E. coli (EIEC), c) enterotoxigenic *E. coli* (ETEC) and d) enterohemorrhagic *E. coli* (EHEC) or *E. coli* O157:H7. The main four types of diarrheagenic *E. coli* have been associated with foodborne illness (Reed, 1994a). There is also a fifth category of diarrheagenic *E. coli*, enteroadherent *E. coli* (EAEC) which are identified by their properties of adherence to HEp-2 cells (Levine, 1987). Bhan et al. (1989b) divided EAEC into three classes by their adherence patterns to HEp-2 cells which are 1) localized adherence (EAEC-L), 2) diffuse adherence (EAEC-D) and 3) aggregative adherence (EAEC-Agg or EAggEC). EAEC-Agg has been associated with acute and persistent diarrhea in children (Vial et al., 1988; Bhan et al., 1989a, 1989b; Fang et al., 1995). However, *E. coli* O157:H7 will be the primary pathogenic *E. coli* discussed.

Typically, infections of *E. coli* O157:H7 can result in three types of illness: 1) hemorrhagic colitis, 2) hemorrhagic uremic syndrome (HUS) and 3) thrombotic thrombocytopenic purpura (TPP). Hemorrhagic colitis is characterized by sudden onset of severe crampy abdominal pain, followed within 24 hr by watery diarrhea which becomes grossly bloody. Onset time is 3 to 7 days with a 2 to 9 day duration. Vomiting is common but fever is rare. Hemolytic uremic syndrome predominantly occurs in children and is a leading cause of acute kidney failure. Individuals with HUS experience bloody diarrhea and substantial morbidity. They often require dialysis and blood transfusions. Central nervous system disease may develop which is characterized by frequent seizures and prolonged coma. Death may result. Thrombotic thrombocytopenic purpura is similar to HUS; however, a dominant feature is central nervous system involvement, accompanied by fever. Abdominal pain and major gastrointestinal hemorrhaging can occur prior to onset of TPP. Patients often develop blood clots in the brain, and death frequently results (Doyle and Padhye, 1989).

Escherichia coli O157:H7 has been recognized as an important cause of foodborne illness, with outbreaks being reported in the U.S.A, Canada and United Kingdom (Doyle, 1991). Bean and Griffin (1990) reported that several ground beef associated outbreaks of hemorrhagic colitis occurred in the U.S. in 1982. *E. coli* O157:H7 was the disease causing organism for these outbreaks. Since 1982, other food related outbreaks have been associated with the consumption of ground beef, Mexican food containing ground beef, unpasteurized apple cider, unpasteurized milk, water, raw potatoes, turkey roll and mayonnaise (Bean and Griffin, 1990; Todd, 1992; Marks and Roberts, 1993). Willshaw et al. (1994) linked verocytotoxin-producing *E. coli* O157 isolated from raw beef burger to an outbreak of diarrhea, hemorrhagic colitis and HUS in Britian. The U.S. Department of Agriculture (USDA) Economic Research Service estimated the annual medical costs and productivity losses associated with *E. coli* O157:H7 to be from 216 to 580 million dollars. It was identified as the fourth most costly foodborne disease, behind *Toxoplasma gondii* (a parasite), *Salmonella* and *Campylobacter* (Marks and Roberts, 1993). In a two year study of 10 hospitals throughout the U.S., *E. coli* O157:H7 was isolated from 99 (0.4%) of 26,239 stool specimens compared with *Campylobacter* (5.7%), *Salmonella* (3.4%) and *Shigella* (3.6%). *E. coli* O157:H7 was isolated more frequently at hospitals in northern and western states than in southern states (Griffin et al., 1993).

Both dairy and beef cattle have been identified as reservoirs for *E. coli* O157:H7 and isolates have been obtained from both healthy and sick animals. *E. coli* O157:H7 has also been reported to colonize the ceca of chickens. Its isolation from retail samples of pork, lamb and turkey suggest that it may also colonize the intestinal tract of these animals. Therefore, a potential vector for dissemination of *E. coli* O157:H7 to the human population is food contaminated by feces (Madden, 1994).

Although *E. coli* O157:H7 has the potential to contaminate meat products it is more heat sensitive than *Salmonella*. *E. coli* O157:H7's sensitivity to heat in meat is affected by the type of meat, the meat's composition and the storage temperature of the meat. At 57°C, D-values for lean (2% fat) and fatty (30.5%) beef are 4.1 and 5.3 min. D-values at 63°C are 0.3 and 0.5 min in lean and fatty beef (Line et al., 1991). Ahmed et al. (1995) reported D-values at 60°C for ground chicken (3 and 11% fat) of 0.38 and 0.55 min, ground turkey (3 and 11% fat) of 0.55 and 0.58 min, ground beef (7, 10 and 20% fat) of 0.45, 0.46 and 0.47 min and pork sausage (7, 10 and 30% fat) of 0.37, 0.46 and 0.55 min, respectively. Jackson et al. (1996) observed that in ground beef patties (22.1% fat), *E. coli* O157:H7 was more sensitive to thermal destruction when patties were held at 15°C for 9 hr than when they were stored at -18°C for 8 days. Holding patties at 21 or 30°C for 4 hr immediately prior to cooking increased the organism's sensitivity to heat in patties stored at both -18 and 15°C. Cooking meat products to an internal temperature of 70°C should destroy any *E. coli* O157:H7 present in raw meat. However, there is still concern about food-to-food and human-to-food contamination. *E. coli* O157:H7 is extremely virulent, a small dose can pose a significant hazard depending upon the strain of the organism and the health of the infected individual. Therefore, the USDA, Food Safety Inspection Service (FSIS) has determined that the presence of any amount of the organism in/on a meat or poultry product will result in the product being deemed as adulterated (USDA, FSIS, 1996).

Salmonella

Various aspects of *Salmonella* have been reviewed. D'Aoust (1989) examined the physiology, pathogenesis, epidemiology and control of *Salmonella*. Marth (1993) reviewed the growth and survival of *Salmonella* species in the presence of sodium chloride. Reed (1993b) briefly discussed food implicated as vectors for salmonellosis and the control of *Salmonella* in foods. Tietjen and Fung (1995) reviewed the history and incidence of foodborne salmonellosis, methodology for detecting foodborne *Salmonella*, applications of detection methods in terms of food safety and systems to prevent and control *Salmonella* in food. Foster (1995) focused on low pH adaptation and the acid tolerance response of *S. typhimurium*. Rowbury (1995) also assessed the environmental factors influencing acid tolerance and sensitivity in *Salmonella*. The growth requirements, disease characteristics and epidemiology of *Salmonella* will be discussed in this review.

The genus *Salmonella* consists of over 200 serovars, most of which exhibit pathogenicity. In the U.S., *Salmonella* is generally classified by the Ewing system which recognizes three species: *Salmonella typhi, Salmonella choleraesuis* and *Salmonella enteriditis*. Two additional identification schemes are the Kauffman-White antigenic scheme and the Le Minor scheme based on numerical taxonomy and DNA-relatedness studies. The Kauffman-White scheme is divided into 5 subgenera (I-V) with identification being based on somatic (O), capsular (V_i) and flagellar (H) antigenic profiles. The Le

Minor scheme consists of a single species, *S. choleraesuis*, subdivided into seven subspecies which includes: *choleraesuis*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, *bongori* and *indica* (D'Aoust, 1989).

Salmonella is a Gram-negative facultative anaerobe, commonly associated with poultry. However, it is not host specific with primary reservoirs being animals and humans. Cells are straight rods usually motile with peritrichous flagella. Growth occurs at 5 to 47°C, with an optimum temperature range being 35 to 37°C. The optimum pH for growth is 6.5 to 7.5 with a range of 4.0 to 9.0. Variations in pH sensitivity are dependent upon the nature of the acidulant, incubation temperature, serovar, aerobicity and characteristics of the growth media. The minimum a_w for growth is 0.94. Heat resistance varies with a_w and the nature of solutes in the heating menstruum (D'Aoust, 1989). The maximum heat resistance is 480 min at 70°C and the minimum is 1 min at 60°C. Species are unable to tolerate salt, 3 to 4% is inhibitory and 9% is bactericidal. The survival of *Salmonella* spp. in various media and food containing > 5% salt is affected by temperature. The organisms tend to survive longer in the presence of salt (5 to 25%) when subjected to 0 to 8°C than when stored at 18 to 37°C (Marth, 1993).

Salmonella can cause four types of clinical disease syndromes: 1) enteric fever, typified by typhoid fever, 2) paratyphoid (typhoid like), 3) gastroenteritis and 4) septicemia. Typhoid (enteric fever) is characterized by malaise, anorexia and headache followed by fever. Pyrexia prevails for several weeks with patients possibly becoming delirious. Septicemia is usually evident after 10 or more days, with *Salmonella* being isolated from blood and urine. After onset of illness *Salmonella* is not observed in stool samples until 3 weeks after infection (D'Aoust, 1989).

Gastroenteritis symptoms include abrupt onset of nausea, fever, headache, vomiting, nonbloody diarrhea, dehydration and abdominal cramps. Onset occurs 8 to 72 hr after consuming contaminated food or water. Infective doses range from 10^2 to 10^{10} organisms/g and depend on the strain's virulence and the host's age and immune status. Gastroenteritis is usually self-limiting lasting 1 to 7 days, with organisms being excreted for many weeks after symptoms subside. Individuals with underlying diseases such as sickle cell anemia, liver or gall bladder diseases, malignancies or acquired immunodeficiency syndrome (AIDS) are prone to septicemia (D'Aoust, 1989).

In the years, 1973 to 1985 and 1983 to 1987 *Salmonella* accounted for 51 and 57% of the foodborne illness cases caused by bacterial pathogens, respectively (Bean and Griffin, 1990; Bean et al., 1990). The annual incidence of salmonellosis in the U.S. were 17.4 and 11.8 cases/100,000 people in the years 1981 to 1982 and 1968 to 1974, respectively. Approximately 9,000 deaths are associated with the 2,000,000 salmonellosis cases reported annually in the U.S.

Bean and Griffin (1990) identified major food vehicles for salmonellosis and their percentage of outbreaks as beef (16%), turkey (8%), chicken (6%), ice cream (6%), pork (5%), dairy products (5%) and eggs (3%). Other vehicles have included water, Brazilian black pepper, aspic glazing and edible offals (e.g. liver). In many cases, food contamination has been linked to cross-contamination by handling personnel, equipment or other contaminated foods. Other contributing factors in salmonellosis outbreaks include inadequate cooking of raw food ingredients and slow cooling and improper hot storage of cooked foods. Reed (1993b) outlined four principles to prevent salmonellosis in food service institutions 1) cooking foods thoroughly and serving them hot (> 60°C), 2) using sanitary practices to prevent cross-contamination, 3) prompt cooling/freezing of small portions of prepared foods and 4) complete reheating (≥ 74°C) of stored refrigerated foods. Tietjen and Fung (1995) indicated that internal temperatures should reach at least (71°C) for beef and pork and (85°C) for poultry to prevent salmonellosis.

The USDA, FSIS (1996) has proposed using *Salmonella* as an indicator pathogen in the Pathogen Reduction Program for raw meat products. Initially, FSIS will take approximately 250 samples per establishment per year. Sampling rates will vary as each establishment develops a history of compliance. The performance standards are presented in Table 2-4.

Table 2-4. USDA, FSIS pathogen reduction performance standards

Class of product	Performance standard (percent positive for *Salmonella*) (%)	Number of samples tested	Maximum number of positives to achieve standard
Steers/Heifers	1.0	82	1
Cows/Bulls	2.7	58	2
Ground Beef	7.5	53	5
Fresh Pork	NA[a]	NA	NA
Broilers	20.0	51	12
Hogs	8.7	55	6
Ground Turkey	49.9	53	29
Ground Chicken	44.6	53	26
Turkeys	NA	NA	NA

[a] Not available at this time.
Adapted from USDA, FSIS (1996).

Yersinia enterocolitica

Schiemann (1989) reviewed the growth, survival and disease characteristics and epidemiology of *Y. enterocolitica.* Andersen et al. (1991) discussed different aspects of the epidemiology of *Y. enterocolitica.* Pathogenic *Y. enterocolitica*, their reservoirs for human infection and prevention and control measures to reduce food contamination were reviewed by Kapperud (1991). Farrag and Marth (1992) reviewed *Y. enterocolitia's* history, growth requirements, disease characteristics, outbreaks, occurrence in foods and reservoirs and discussed the potential for controlling it with the lactoperoxidase system in milk products. Schofield (1992) discussed *Y. enterocolitica's* presence and significance in chilled foods. Reed (1994c) discussed yersiniosis, foods implicated as outbreak agents and methods of preventing food contamination and controlling *Y. enterocolitica* in foods. This review will discuss *Y. enterocolitica's* growth requirements, disease characteristics, epidemiology, survival in meat products and presence in dairy processing facilities.

Yersinia enterocolitica is a true psychrotroph capable of growth under refrigerated temperatures. Growth range is 2 to 45°C, with optimum at 32 to 34°C. It fails to grow on standard enteric agar at 35 to 37°C, but will grow at 22 to 25°C. Poor growth at high temperatures is primarily due to increased toxicity of selective agents combined with increased requirements for calcium. Cells are Gram-negative, straight rods or cocci. They are nonmotile when grown at 37°C, but motile with peritrichous flagella when grown at 30°C. *Y. enterocolitica* is resistant to freezing and can survive in frozen foods for long periods of time. It is intolerant of high temperatures, D values at 62.8°C are 0.24

to 0.96 min, which results in destruction during standard pasteurization (Schiemann, 1989).

Yersinia enterocolitica exhibits slow growth and is a poor competitor against spoilage bacteria. It stops growing when competing bacteria reach the stationary growth phase. This is not the result of nutrient depletion, oxygen limitation or accumulated toxic metabolites, but is attributed to a phenomena described as "metabolic crowding." Low temperature enrichment (4 to 15°C) is effective for isolating initially low numbers of *Y. enterocolitica* from mixed cultures. Low temperatures reduce the growth rate of microbial antagonists and allow *Yersinia* to reach higher numbers before the density of competing bacteria becomes restrictive (Schiemann, 1989).

Enteric infections of *Y. enterocolitica* commonly cause diarrhea, fever, headache, intense abdominal pain, nausea and vomiting. Enteric infections are generally self-limiting and involve the ileoceccal region of the gastrointestinal tract. *Y. enterocolitica* is often isolated from appendixes when patients have been diagnosed with false appendicitis (ileitis). Other intestinal infections can involve the mesenteric lymph nodes, colon and small intestine. Extraintestinal infections include arthritis, septicemia, erythema nodosum (a cutaneous granuloma), skin infection, eye infection, endocarditis, thyroid disorders, liver disease, respiratory infection, neurological complications and muscle abscess (Schiemann, 1989).

Yersinia enterocolitica was identified as a foodborne pathogen in the early 1970s; however, there have only been a few major outbreaks and occasionally sporadic occurrences over the past decades (Doyle, 1992). The largest foodborne outbreak of yersiniosis in the U.S. was associated with pasteurized milk. Pigs were implicated as the source via milk crates. Outdated milk was transported in crates to a pig farm, the crates were then washed and used to transport fresh milk to retail outlets. It was presumed that mud, contaminated with *Y. enterocolitica*, got onto the crates. *Yersinia* survived cleaning and 4°C storage, was transferred onto the new milk cartons, then into the milk and multiplied to infectious doses under refrigeration.

Another potential source of infection from *Y. enterocolitica* is fresh dry sausages such as soft pork spread, Berlin knackwurst and cervalet (Kleemann and Bergann, 1996). The survival of *Y. enterocolitica* O:9 was evaluated in each of the fresh dry sausages, without the addition of starter cultures and stored at 3-5°C or 13-16°C. With inoculum loads of 4.69 $\log_{10}$ CFU/g, *Y. enterocolitica* survived in soft pork spread (pH, 5.7; sodium chloride, 3.75%; moisture, 47.9%; and a_w, 0.96) for 73 days at 3-5°C and 35 days at 13-16°C. In Berlin knackwurst (pH, 5.7; sodium chloride, 2.41%; moisture, 53.1%; and a_w, 0.96), the inoculum was 5.3 $\log_{10}$ CFU/g and survival was 60 days at 3-5°C and 46 days at 13-16°C. Similarly, for cervalet (pH, 5.85; sodium chloride, 2.45%; moisture, 45.2%; and a_w, 0.95), the inoculum was 5.0 $\log_{10}$ CFU/g and survival was 60 days at 3-5°C and 45 days at 13-16°C.

In vitro studies have shown sodium nitrite, sodium nitrate or potassium nitrate (5 to 20 mg/ml) to exhibit only minimal antimicrobial activity against *Y. enterocolitica* (de Giusti and de Vito, 1992). However, *in vivo* studies utilizing ground pork, nitrite and nitrate (150 to 350 mg/kg) were substantially more effective than in the *in vitro* studies. The higher activity in the *in vivo* system was likely the result of competition from

Streptococcus liquefaciens, *S. marcescens*, *E. coli* and *Proteus mirabilis* which were also isolated from the meat samples.

Asplund et al. (1993) evaluated the survival of *Y. enterocolitica* in fermented sausage manufactured with different levels of nitrite and different starter cultures. After 28 days, *Y. enterocolitica* was not detected in sausages that were inoculated with 10^5 CFU/g *Y. enterocolitica* and containing 80 to 120 mg/kg sodium nitrite. Sausages manufactured without and with 50 mg/kg sodium nitrite harbored *Y. enterocolitica* throughout the 35 day test period. In the absence of nitrite, *Lactobacillus pentosus* and *Lactobacillus plantarum* were more inhibitory to *Y. enterocolitica* than *Pediococcus acidilactici*. After 35 days, *Y. enterocolitica* counts were < 2.0, 2.3 and 5.9 $\log_{10}$ CFU/g in sausages fermented with *L. pentosus*, *L. plantarum* and *P. acidilactici*, respectively.

Yersinia enterocolitica has been isolated from most domesticated livestock and various forms of wildlife. However, these serovars are rarely recognized as human pathogens. Swine has been identified as the principal reservoir of virulent human pathogenic strains. Pathogenic *Y. enterocolitica* are frequently isolated (50% of samples) from the throat of healthy pigs at the time of slaughter. Contact with swine has been documented as a vehicle for human infection. However, the only pork products recognized as a reservoir for pathogenic *Y. enterocolitica* are pork tongues and ground pork. It has been isolated from fresh meats, raw milk, pasteurized milk, vacuum packaged meats, seafoods, vegetables, poultry and water (Schiemann, 1989; Reis-Tassinari et al., 1994). Chocolate milk, coleslaw, tofu, bean sprouts, pasteurized milk and chitterlings have been implicated in yersiniosis outbreaks (Adams et al., 1991; Reed, 1993c).

Andersen et al. (1991) reported that the number of pork carcasses contaminated with *Y. enterocolitica* could be reduced by utilizing a mechanical bung cutter and enclosing the rectum and anus in a plastic bag prior to evisceration when compared to traditional manual evisceration. Manual evisceration resulted in surface contamination of 26% on the medial hind limb and 13% on the split sternum and surrounding area while mechanical bunging exhibited only 2% contamination on both sites. They also reported that *Y. enterocolitica* positive minced meat samples were obtained from retail butcher shops where positive minced pork samples were also obtained. This observation identified cross-contamination as a potential means of human infection.

In addition to its contamination of foods, *Y. enterocolitica* has been isolated from food processing environments. Pritchard et al. (1995) surveyed thirty dairy processing plants for the presence of *Yersinia* spp. In ten (33.3%) of the plants one or more of the tested sites were positive. A total of 20 of 347 (5.8%) of the sites tested were positive for one or more species of *Yersinia*. From the 20 positive sites, 17 (85%) were positive for *Y. enterocolitica*. Positive sites were coolers/freezers, raw milk receiving areas and drains around machinery including an ice maker, a case washer, holding tanks, a filler machine and a packaging area.

Reed (1994c) discussed four methods for protecting food from contamination with *Y. enterocolitica*. 1) Handle pork and other foods in a sanitary manner, taking special care to avoid contamination of ready-to-eat foods. 2) Thoroughly cooking/reheating foods to (74°C) before consumption or sale. 3) Washing/sanitizing hands prior to food handling and eating and after handling raw pork and other meats. 4) Protect water supplies from contamination with human and animal fecal waste.

Listeria monocytogenes

Lovett (1989) discussed the physiology, disease characteristics and epidemiology of *L. monocytogenes*. Donnelly (1990) discussed the history of *L. monocytogenes*, food linked listeriosis epidemics, the incidence of *Listeria* spp. in foods, regulatory responses to *Listeria* and methods of preventing and controlling foodborne *Listeria*. Johnson et al. (1990) reviewed *L. monocytogenes* and other *Listeria* spp. in meat and meat products with emphasis being placed on the isolation, incidence and levels of *Listeria* contamination in meat and poultry, the prevalence of *Listeria* in meat plants and the survival of *Listeria* on fresh meats, during meat processing and in processed meat products. Schofield (1992) briefly discussed the potential for human listeriosis from unpreserved ready-to-eat chilled foods. Marth (1993) reviewed the growth and survival of *L. monocytogenes* in the presence of sodium chloride. Reed (1993b) discussed foods that *L. monocytogenes* has been isolated from, listeriosis, foodborne outbreaks and prevention and control of *Listeria* in foods. Rocourt (1994) discussed the taxonomy, identification, typing, ecology, virulence, detection methods, illness and epidemiology associated with *L. monocytogenes*. Madden (1994) discussed various listeriosis outbreaks, food associated with the outbreaks and the controversy around the "zero tolerance" policy for *L. monocytogenes* in ready-to-eat, cooked meat and poultry products. McNamara (1994) reviewed several outbreaks and

USDA, FSIS, Microbiology Division's surveillance and monitoring programs for raw and ready-to-eat meat and poultry products. Marsden (1994) discussed industry perspectives toward *L. monocytogenes* and methods of reducing its incidence and controlling its growth in raw and cooked meat and poultry. This review will focus on *L. monocytogene's* physiology, pathogenesis, epidemiology and contamination of meat products, meat processing facilities and the environment.

Listeria monocytogenes is Gram-positive, though older cultures tend to lose staining ability. Cells are short rods with rounded ends occurring singly or in short or long chains. Cells grow under aerobic and anaerobic conditions. Optimum temperature of growth is 30 to 37°C with a range of 1 to 45°C. Optimum pH is 6.0 to 9.0 and the pH range is 4.5 to 9.5. Resistance to heat varies with medium. *L. monocytogenes* is halophilic with growth in up to 12% NaCl and survival at 30% NaCl (Marth, 1993). Salt tolerance is temperature dependent in that reduced temperature results in increased tolerance. Serovars are sensitive to a variety of antibiotics. Biochemical characteristics include lypolytic; CAMP positive with *S. aureus*; CAMP negative with *R. equi*; L-rhamnose positive; D-xylose negative; esculin positive; and hemolytic (Lovett, 1989).

The enteric phase of listeriosis may or may not cause symptoms. When symptoms occur they are similar to transitory flu-like symptoms and include malaise, diarrhea and fever. The infective dose is currently unknown. Incubation time may be as low as one day, with one to several weeks being common in adults. Virulent strains are capable of producing septicemia, which is diagnosed when *L. monocytogenes* is isolated from a patient's blood without documented infection elsewhere. Septicemia is characterized by fever and nonspecific complaints which may include fatigue, malaise, vomiting, pain and diarrhea. The mortality rate associated with septicemia is approximately 30% with new born, immunocompromised or debilitated patients. Septicemia allows *L. monocytogenes* access to the central nervous system, heart, spleen, lymph nodes, bones and other organs. The most common form of listeriosis involving the central nervous system is meningitis. It develops primarily in newborns and the elderly and has a fatality rate of 70% in untreated or late treated cases (Lovett, 1989). Septicemia also allows *L. monocytogenes* access to the fetus in pregnant women. The outcome can be abortion, stillbirth or neonatal septicemia depending on the stage of pregnancy when the organism invades the fetus (Rocourt, 1994).

Listeria monocytogenes has been reported as a foodborne pathogen since 1981. This organism's economic and public health impacts make it one of the most important foodborne pathogens. There are approximately 1,700 listeriosis cases in the U.S. annually (Donnelly, 1990). Bean and Griffin (1990) reported *L. monocytogenes* as having the highest death-to-case ratio, 317/1,000, among the foodborne pathogens. It was followed by *C. botulinum* with a death-to-case ratio of 192/1,000. As a result of the high mortality rate the FDA and USDA Food Safety Inspection Service have imposed a zero tolerance for the presence of *L. monocytogenes* in dairy products and ready-to-eat processed foods (Donnelly, 1990; Madden, 1994; McNamara, 1994; Reed, 1994b).

Listeriosis outbreaks from 1981 to 1985 have been associated with pasteurized whole and 2% milk, Mexican-style soft cheese and coleslaw (Bean and Griffin, 1990). *L. monocytogenes* has been isolated from a variety of raw foods which include milk, poultry carcasses, lamb, beef, pork, celery, tomatoes, lettuce, shrimp, lobster, crab meat, langostino and finfish (Lovett, 1989). *Listeria* spp. including *L. monocytogenes* have been isolated from a variety of cooked processed meat products which include frankfurters, bologna, luncheon meats, corned beef, ham, smoked fish, sliced turkey and chicken products and pre-cooked, chilled chicken (Johnson et al., 1990; Kerr et al., 1990; Tiwari and Aldenrath, 1990; Dillion et al., 1992; Grau and Vanderlinde, 1992).

Wang and Muriana (1994) isolated *L. monocytogenes* from 6 of 20 brands frankfurters purchased from retail supermarkets in West Lafayette, Indiana. There was an overall incidence of 7.5% (7 positives per 93 packages) for 19 brands of franks. The twentieth brand exhibited an incidence of 71% (17 positives per 24 packages). Levels ranged from 4.3 to 27.6 most probable numbers/package. *L. monocytogenes* was isolated from the liquid exudates in the packages but not the interior of the frankfurters, indicating that its presence was likely the result of post processing contamination.

Franco et al. (1995) investigated the principle sources of *Listeria* spp. contamination in poultry meat in a poultry processing plant in Spain. *Listeria* spp. contaminated 96% of the drumstick skin samples, 64% of the drumstick meat samples, 84% of the wing skin samples, 68% of the wing muscle samples, 80% of the breast samples and 0% of the liver samples taken. Maximum levels of *Listeria* were 4.2 log CFU/g which were observed on the drumsticks. The maximum counts observed on the wings were 2.8 log CFU/g. Evaluation of environmental contamination revealed that 77% of the conveyor belt surface samples, 61% of the table surface samples and 60% of the circular saw samples were contaminated with hemolytic *Listeria*. In a similar study, analysis of gloves and workers hands revealed that up to 59% of the workers on the final part of the production line carried *Listeria* spp. (Genigeorgis et al., 1989). Franco et al. (1995) hypothesized that *Listeria* contamination was acquired on the farm and carried on the skin of the poultry, mainly on the drumstick. During processing the contamination was distributed over the carcasses and throughout the processing facility.

The 63°C D-values for *L. monocytogenes* in 20 and 30.5% fat ground beef are 0.5 and 1.1 min, respectively (Fain et al., 1991). Hansen and Knochel (1996) reported $D_{64°C}$ and Z values of 1.4 to 1.7 min and 6.5 to 6.9°C, respectively, for *L. monocytogenes* during rapid and slow heating of *sous vide* cooked beef. In ground pork, $D_{60°C}$ and Z values have been reported to be 1.14 min and 5.05°C, respectively (Ollinger-Snyder et al., 1995). These reports suggest that *L. monocytogenes* should be destroyed in processed meat cooked to 70°C. However, in ground pork (15% fat) heated to 60°C, singular applications of sodium chloride (0.5 to 3%), dextrose (1%) and a phosphate mixture (0.4%) protected *L. monocytogenes* from thermal destruction. A combination of 2% sodium chloride, 1% dextrose, 0.4% phosphates, 0.055% erythorbate and 0.0156% sodium nitrite provided more thermal protection than the singular application of each additive (Yen et al., 1991). This suggests that common curing ingredients may promote the survival of *Listeria* in processed meat products.

Listeria monocytogenes has been isolated from all of the major meat species of livestock and a wide variety of wild and domestic animals. Estimates indicate that as many as 5% of human populations may excrete *L. monocytogenes* at a given time. It is capable of surviving in many environmental situations. It is also capable of surviving various cheese processing methods and production of nonfat dry milk. *L. monocytogenes* survives 14 days of storage in refrigerated (4°C) ground beef in oxygen permeable and impermeable packaging. It has been isolated from animal feces, fodder, dry straw, fertile soil, clay soil and tile surfaces after 30 days and up to two years after initial contamination. It is often found in sewage plant effluent at 10^3 to 10^4 CFU/ml and is capable of survival in sewage sludge for many months. *L. monocytogenes* often contaminates canals and lakes. Contamination is probably the result of receiving sewage effluent from nearby cities and towns (Lovett, 1989).

Staphylococcus aureus

The growth, physiology, survival, enterotoxin production, disease characteristics and epidemiology of *S. aureus* were reviewed by Bergdoll (1989). Marth (1993) briefly discussed the effects of sodium chloride on the growth and survival of *S. aureus*. Reed (1993a) discussed *Staphylococcal* food poisoning and principles of controlling food contamination with *S. aureus*. This review will focus on the growth characteristics, disease characteristics and control of *S. aureus*.

Staphylococcus aureus is a ubiquitous organism generally associated with the skin, cuts and nasal areas of healthy humans and animals. Approximately 30 to 50% of humans are carriers with 15 to 30% being persistent carriers. It is an opportunistic pathogen of humans and animals. It is a Gram-positive, nonmotile facultative anaerobe. Cells are commonly found singly, in pairs or in clusters. *S. aureus* exhibits optimum growth at 35 to 37°C, with a range of 5 to 48°C. The optimum pH is 7.0 to 7.5 with a range of 4.2 to 9.3. The minimum a_w is 0.83 for aerobic growth and 0.90 for anaerobic growth (Bergdoll, 1989). *S. aureus* is halophilic with growth in up to 12% NaCl, inhibition occurring at 15 to 20% NaCl and bactericidal effects occurring at 22 to 25% NaCl (Marth, 1993). Cells are heat sensitive, with death occurring at pasteurizing temperatures.

Staphylococcus aureus produces a variety of toxins. The most important in food poisoning are a group of heat stable single polypeptide enterotoxins: A, B, C_1, C_2, D and E. The LD_{50} for humans are 20 ng/kg for A and 500 ng/kg for B. The emetic dose (ED_{50}) is 5 to 20 μg/animal. They appear in cultures as early as 4 hr, increase proportionally in the log phase, but are produced in all stages of growth. Enterotoxin production in food is dependent upon food composition, physical nature, pH, moisture content, a_w and storage time, temperature and atmosphere. The optimum temperature for toxin production is 37°C. Toxin production is reduced at 30 and 25°C and stops at 13 to 15°C. In 0.2 M sodium phosphate the optimum pH for toxin B production is 7.0 to 7.5. At pH 6.0 and 8.0 toxin production is reduced considerably. In casein hydrolysate, maximum amounts of toxin were produced at initial pHs of 6.0 and 6.5. The pH range for enterotoxin production is 5.15 to 9.0. Enterotoxin production has been reported at water activities as low as 0.86. However, there is an interaction between pH and a_w. When media pH is low, the minimum a_w for enterotoxin production is high (Bergdoll, 1989).

Staphylococcus aureus enterotoxins are superantigens which stimulate 1 in 5 T cells, while typical polypeptide antigens stimulate 1 in 10,000 T cells. The superantigens

(SAg) bind to the outside of the antigen binding groove of the major histocompatibility complex (MHC) class II molecule. It then binds to the variable region of the β-chain of the T cell receptor (TCR) forming a ternary complex of MHC:SAg:TCR. Massive T cell stimulation occurs which results in the production of large quantities of cytokines such as interferon-gamma, tumor-necrosis factor and various interleukins. These cytokines likely induce the physical response associated with *S. aureus* food poisoning (Lindsay, 1997).

A typical reaction to *S. aureus* intoxication begins with nausea 1 to 6 hr after eating food contaminated with enterotoxin. Severe abdominal cramps frequently develop and are followed by severe vomiting at 5 to 20 min intervals for 1 to 8 hr. Vomitus is blood streaked in 10 to 15% of cases. Diarrhea often accompanies vomiting and continues for several hr. In some cases, blood is observed in the stool. During the acute stage, temperature is normal to subnormal, pulse increases noticeably, cold sweats occur, prostration is severe and shock often develops. Headaches are mild and of short duration. Muscular cramping occurs in the legs. Dehydration often occurs. Acute symptoms typically last 1 to 8 hr and complete recovery occurs in 1 to 2 days. The only cure is bed rest and good fluid balance. Humans do not develop immunity to further attacks (Bergdoll, 1989).

Foodborne outbreaks have involved milk, cream, custard- and cream- filled baked goods, cheese, canned corned beef, dried lasagna and chicken-, tuna-, ham- and potato-salads. Many cases of food poisoning by *S. aureus* have been linked to food handlers that carry the organism in facial or hand lesions or nasal or throat passages. *S. aureus* is a common contaminant of fresh meat and other animal products, however, it is rarely involved in foodborne illness from these sources because of its sensitivity to heat and inability to compete with other organisms. In situations where foodborne illness is related to raw product contamination, there are typically large initial levels of *S. aureus* involved (Bergdoll, 1989).

In today's food industry, the greatest risk of processed meat contamination with *S. aureus* is handling cooked products. For example, cooked turkey rolls, ham and roast beef are often packaged then cooked at a meat processing facility. These packages are shipped to a deli or sandwich shop where an employee opens the package, cuts off several slices then places the package in a refrigerated display case. If the employee was not wearing gloves, had a minor cut on their hand, sneezed or the slicer was not very clean, the meat product may become contaminated with *S. aureus*. In such a case, if the product were to be temperature abused, there would be very little inhibition against the growth of *S. aureus* and potential foodborne illness.

Reed (1993a) identified six principles for controlling *Staphylococcal* food poisoning. 1) Preventing food handlers from working when they exhibit signs of acute respiratory illness or infected wounds or abrasions. 2) Employees using good personal hygiene. 3) Minimizing handling and direct contact of food to skin. 4) Maintaining food contact surfaces in sanitary condition. 5) Not holding food in the temperature "danger zone" of 7.2 to 60°C. 6) Rapidly cooling leftovers to $\leq$ 7.2°C and discarding or freezing leftovers within 36 hr of original preparation.

CHAPTER 3

THE EFFECTS OF SODIUM LACTATE, GLUCONO-DELTA-LACTONE, DISODIUM ETHYLENEDIAMINETETRAACETATE, NISAPLIN AND THEIR COMBINATIONS ON THE GROWTH OF *ESCHERICHIA COLI* O157:H7

Introduction

As previously discussed in Chapters 1 and 2 the overall purpose of this research was to identify and utilize common preservatives in a meat product to provide shelf-stability. Nisin, sodium lactate, glucono-delta-lactone and ethylenediaminetetraacetic acid (EDTA) were reviewed in detail in Chapter 2 and selected as preservatives to be evaluated for their antimicrobial activity against common foodborne pathogens at optimum temperatures of growth.

Nisin is a bacteriocin produced by certain strains of *Lactococcus lactis*. It has been shown to exhibit antimicrobial activity against *Clostridium botulinum*, *Listeria monocytogenes*, *Listeria ivanovii*, *Listeria innocua*, *Staphylococcus aureus*, *Streptococcus lactis* and *Bacillus licheniformis* in media and in dairy, fish and meat products (Bell and De Lacy, 1985; Somers and Taylor, 1987; Benkerroum and Sandine, 1988; Taylor et al., 1990; Harris et al., 1991; Motlagh et al., 1991; El-Khateib et al., 1993; Fang and Lin, 1994). In pork slurries and meat chunks nisin is effective when pH is 5.1 and less (Rayman et al., 1983; Chung et al., 1989). The antimicrobial activity of nisin toward *L. monocytogenes*, *S. aureus* and *Bacillus* spp. spores was enhanced by low storage temperatures, increased sodium chloride levels and reducing pH to 5.0 (Oscroft et al., 1990; Thomas and Wimpenny, 1996).

Sodium lactate (SL) delays the growth of *L. monocytogenes*, *Pseudomonas fragi*, *Clostridium sporogenes*, *Salmonella typhimurium* and *Escherichia coli* O157:H7 and the toxin production of *C. botulinum* (Maas et al., 1989; Harmayani et al., 1991; Shelef and Yang, 1991; Weaver and Shelef, 1993; Houtsma et al., 1994; Miller and Acuff, 1994; Wederquist et al., 1994). Sodium lactate has also been reported to exhibit synergistic antimicrobial activity with nisin (Buncic et al., 1995) and glucono-delta-lactone (Qvist et al., 1994). The USFDA recognizes sodium lactate as a GRAS ingredient for direct use in human foods (USFDA, HHS, 1995).

Glucono-delta-lactone (gluconic acid lactone, GDL) is commonly used in some fermented sausages at 0.5% and is allowed in Genoa salami at 1.0% (Romans et al., 1977). It is an acidulant and does not impart strong acid flavors to food. El-Shenawy and Marth (1990) observed that 0.4% GDL reduced the growth of *L. monocytogenes* in tryptose broth at 13°C. In reconstituted milk, 1.5% GDL was required to reduce growth at 13°C. When incorporated into Italian style dry sausage at 0.4 or 1.0% GDL reduced the growth of *S. aureus* strain S-6 by 2-3 $\log_{10}$ CFU/ml (Metaxopolous et al, 1981). GDL also exhibits antimicrobial effects in pork liver paté (Madden et al., 1989) and Egyptian fresh sausage (El-Khateib and El-Rahman, 1987). GDL exhibits synergistic antimicrobial activity with nisin (Oscroft et al., 1990) and sodium lactate (Qvist et al., 1994).

Ethylenediaminetetraacetate is a metal chelating agent that functions as both an antioxidant and antimicrobial agent. The antioxidant activity involves binding divalent metal cations which prevents them from oxidizing lipids. In trypticase soy broth, 0.5% EDTA inhibited the growth of *Salmonella typhimurium* and *Salmonella heidelberg* incubated at 37°C for 10 hr (Samuelson et al., 1985). Kraniak and Shelef (1988) reported that EDTA (0.8 to 1.7 mM) inhibited the growth of *S. aureus* strain 196E in BHI broth. EDTA exhibited synergistic antimicrobial effects with nisin (Stevens et al., 1991; Cutter and Siragusa, 1995a, 1995b), lysozyme (Teotia and Miller, 1975; Payne et al., 1994) ascorbic acid, sodium acetate and sodium propionate (Golden et al., 1995) and sodium nitrite (Tompkin et al., 1978, 1979). Disodium EDTA is allowed in cooked sausages at levels of 0.0036% (USFDA, HHS, 1995).

This research was undertaken to determine the effects of sodium lactate, glucono-delta-lactone, disodium EDTA, nisin and their combinations on the growth of *E. coli* O157:H7 at 37°C.

Materials and Methods

Experimental design

Two experimental designs were utilized to evaluate the ability of various preservative combinations to inhibit the growth of *E. coli* O157:H7 ATCC 43895. The preservatives used include SL (natural sodium lactate 60% USP liquid, Purac, Inc., Arlington Heights, IL), GDL (Sigma Chemical Co., St Louis, MO), disodium EDTA (Fisher Scientific, Fair Lawn, NJ) and Nisaplin (Alpin and Barrett Ltd, Dorset, England). Nisaplin is composed of sodium chloride, denatured milk solids and 1 x 10^6 international units (IU)/g nisin. For nisin, 1 IU is 1 μg.

Cooked meat media (Difco Laboratories, Detroit, MI, #0267-01-7) was formulated to contain SL, GDL, disodium EDTA and Nisaplin (NN). The first experiment utilized a 3 X 3 X 3 X 3 factorial design with additives at the following levels: SL at 0, 1.5 and 3.0% (V/V); GDL at 0, 0.25 and 0.5% (W/V); EDTA at 0, 0.372 and 0.745% (W/V), which were 0, 0.01 and 0.02 M respectively; and NN at 0, 0.5 and 1% (W/V), which were 0, 0.0125 and 0.025% nisin respectively. Treatments were inoculated with *E. coli* O157:H7 ATCC 43895 to obtain initial levels of 2 to 3.5 $\log_{10}$ CFU/ml. Two replications consisting of duplicate test tubes for each treatment were performed. The treatments were incubated at 37°C for 7 days with growth being evaluated at days 1, 4 and 7 or until numbers exceeded 10^6. Levels of EDTA used in the first experiment exceeded those allowed in meat products and were therefore reduced to near legal levels in a second experiment.

In the second experiment, cooked meat media (CMM) was formulated to contain SL, GDL, EDTA and NN but with lower EDTA levels than the first trial. A 3 X 3 X 3 X 3 factorial design was used with additives at the following levels: SL at 0, 1.5 and 3.0% (V/V); GDL at 0, 0.25 and 0.5% (W/V); EDTA at 0, 0.0036 and 0.0072% (W/V); and NN at 0, 0.5 and 1% (W/V), which were 0, 0.0125 and 0.025% nisin, respectively. Treatments were inoculated with *E. coli* O157:H7 ATCC 43895 to obtain initial levels of 2 to 3.5 $\log_{10}$ CFU/ml. Two replications consisting of duplicate test tubes for each treatment were performed. The treatments were incubated at 37°C for 3 days with growth being evaluated at days 1, 2 and 3 or until numbers exceeded 10^6. Above 10^6 CFU/ml, treatments were not inhibiting growth and any reductions in numbers would be the result of the organisms entering the death phase of the growth curve.

Cooked meat media preparation

Sodium lactate, GDL and disodium EDTA were incorporated into the CMM prior to autoclaving (15 min at 121°C). Nisaplin was dissolved in sterile 0.12 N HCl and aseptically added to the sterile CMM. Final volume was 10 ml. The treatment pHs varied with the level of GDL in each treatment. The average pH for the GDL levels were 6.0 at 0% GDL and 5.0 to 5.5 at 0.25 and 0.5% GDL. The CMM treatments were heated to 70°C in a water bath and immediately cooled in tap water, to simulate cooking of a meat product. *E. coli* O157:H7 was inoculated into the CMM after it cooled to room temperature.

Inoculum

E. coli O157:H7 was inoculated into LST broth (Difco #0241-15-2) and incubated overnight at 37°C. This resulted in inoculum counts of approximately 10^8. The inoculum was serially diluted to approximately 10^4 in 0.1% peptone water (Difco #1807-17-4). At inoculation, initial levels of each organism were determined by enumerating the treatment combination with no additives. Initial levels ranged from 2 to 3.5 $\log_{10}$ CFU/ml.

Microbial analysis

Enumeration consisted of serial dilutions in 0.1% peptone water to 10^{-6} and a variation of the drop plate method (Swanson et al., 1992) where two 10μl drops of each dilution were placed symmetrically around the perimeter of duplicate petri dishes. Plates were incubated in the upright position at 37°C for 24 hr. EMB Levine (Difco #0005-01-

4) was used as the enumeration media. This technique resulted in an enumeration range of 2-8 $\log_{10}$ CFU/ml. The counts from each pair of duplicate petri dishes were averaged to yield one of the two observations for each treatment within a replication. For statistical analysis, observations with less than 2 $\log_{10}$ CFU/ml of growth were recorded as 1.5 $\log_{10}$ CFU/ml.

Statistical analysis

For day 1 of the SL, GDL, EDTA and NN combinations applied to *E. coli* O157:H7, the statistical analysis utilized the general linear models program (PROC GLM) of SAS (SAS Institute, 1985). Variations in microbial growth were divided according to sixteen treatment effects: replication, GDL, EDTA, NN, SL, GDL*NN, GDL*EDTA, GDL*SL, NN*SL, NN*EDTA, EDTA*SL, GDL*NN*SL, GDL*NN*EDTA, GDL*EDTA*SL, NN*EDTA*SL and GDL*NN*EDTA*SL. After day 1, treatment combinations that exceeded 6 $\log_{10}$ CFU/ml were eliminated from the experiment making such a comparison for all days inappropriate. Comparisons between individual means were performed using the lsmean pdiff statement of SAS. Treatment interactions and differences between means were considered significant at $P \leq 0.05$.

Treatments involved in statistical interactions with less than 10^6 CFU/ml at day 1 were statistically analyzed to evaluate their inhibitory effects over time. In order to be selected and evaluated at each day, treatments had to exhibit less than 10^6 CFU/ml in both replications. This resulted in four observations for each treatment at each day. Variations in growth were accounted for by replication, treatment, day of incubation and treatment by day interaction. Differences in the means of each treatment by day combination were identified using the least squares means pdiff statement. Sources of variation and treatment by day differences were considered significant at $P \leq 0.05$.

Results and Discussion

EDTA at 0, 0.372 and 0.745% (0, 0.01 and 0.02 M)

The probability values ($P > F$) obtained from analysis of variance indicated that when EDTA concentrations were 0, 0.01 and 0.02 M, there were significant ($P < 0.0001$) treatment effects and interactions for GDL, NN, SL and EDTA (Table 3-1). There were no significant ($P > 0.05$) treatment interactions between NN and the other additives. The average counts of *E. coli* O157:H7 for each preservative main effect and preservative combination after 1 day of incubation (37°C) are presented in Appendix A (Tables A-1 and A-2).

Nisaplin exhibited a main effect ($P = 0.0003$). The overall averages for treatments containing NN revealed that as NN increased from 0 to 1.0%, *E. coli* O157:H7 numbers declined from 6.4 to 6.0 $\log_{10}$ CFU/ml (Table A-1). However, a 0.4 $\log_{10}$ reduction is not of practical significance.

Table 3-1. Probability values (P > F) for the effects of glucono-delta-lactone, Nisaplin, disodium ethylenediaminetetraacetate, sodium lactate and their combinations on *Escherichia coli* O157:H7 growth after incubation for 1 day at 37°C

Source of variation	P > F 0, 0.372 and 0.745% EDTA	P > F 0, 0.0036 and 0.0072% EDTA
replication	0.0001	0.0073
GDL[a]	0.0001	0.0001
NN[b]	0.0003	0.0036
EDTA[c]	0.0001	0.0120
SL[d]	0.0001	0.0001
GDL*EDTA	0.0001	0.1388
GDL*NN	0.2995	0.0047
GDL*SL	0.0001	0.0001
NN*EDTA	0.2942	0.7042
EDTA*SL	0.0001	0.2207
NN*SL	0.2871	0.0628
GDL*EDTA*SL	0.0001	0.2895
GDL*NN*SL	0.2539	0.0004
NN*EDTA*SL	0.4396	0.2253
GDL*NN*EDTA	0.7418	0.9834
GDL*NN*EDTA*SL	0.5545	0.8098

[a] GDL - glucono-delta-lactone.
[b] NN - Nisaplin.
[c] EDTA - disodium ethylenediaminetetraacetate.
[d] SL - sodium lactate.

The lack of an interaction between EDTA and NN disagrees with Stevens et al. (1991) who observed that individual applications of 0.745% EDTA and 50 μg nisin/ml (0.005% nisin) did not reduce *E. coli* O157:H7 populations while their combination decreased populations by 6.9 $\log_{10}$ CFU/ml. The different results in this study was probably caused by different incubation/exposure times. Stevens et al. (1991) utilized an incubation/exposure time of 1 hr at 37°C, after which cells were harvested by centrifugation, washed twice with cell buffer and transferred to pour plates of brain heart infusion agar. The current study utilized an incubation/exposure time of approximately 24 hr at 37°C. Nisin and pediocin have been shown to cause immediate reductions in the numbers of *S. lactis*, *S. aureus* and *L. monocytogenes* on fresh meat, however during storage the bacteriocins did not prevent growth (Chung et al., 1989; Nielsen et al., 1990; Motlagh et al., 1992; El-Khateib et al., 1993; Fang and Lin, 1994). Similarly, the extended exposure/incubation time in this study may have allowed initially injured cells to repair themselves and proliferate, thus masking any EDTA*NN interactions that may have occurred initially.

For 1 day of incubation samples, the interaction of EDTA, GDL and SL treatment groups was significant ($P < 0.0001$) as shown in Table 3-1. Figure 3-1 presents the mean $\log_{10}$ values for *E. coli* O157:H7 counts as a function of EDTA, GDL and SL levels. These values suggested that the inhibitory effect of GDL and SL combinations was greater when EDTA was included in the mixture (Fig. 3-1, B & C) than when only GDL and SL (Fig. 3-1, A) were combined. As EDTA increased from 0.372 to 0.745%, the inhibitory effects increased. In addition, the combination of 0.745% EDTA with 3.0% SL appeared

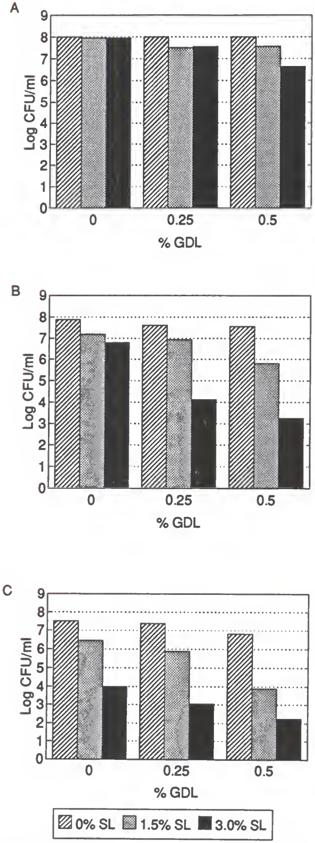

Fig. 3-1—Mean $\log_{10}$ values of *Escherichia coli* 0157:H7 grown in various levels of sodium lactate (SL), glucono-delta-lactone (GDL) and disodium ethylenediaminetetraacetate (EDTA) for 1 day at 37°C (A - 0% EDTA; B - 0.372% EDTA; C - 0.745%

to be almost as inhibitory as some three way combinations. Thus, these differential responses of *E. coli* O157:H7 growth to combinations of additives resulted in the significant interaction of EDTA, GDL and SL. These data indicated that the inhibitory effects of GDL and SL on *E. coli* O157:H7 were enhanced by the levels of EDTA used in this study.

Stevens et al. (1991) hypothesized that EDTA binds with magnesium ions in the lipopolysaccharide layer on the outer membrane of Gram-negative bacteria and thereby increases membrane permeability and cell susceptibility to antimicrobial agents. The binding action of EDTA toward magnesium ions on the cell membrane may have been the cause of EDTA's interaction with GDL and SL.

Combinations of GDL and SL exhibited a slight increase in antimicrobial activity as the concentrations of each preservative increased (Fig. 3-1, A). This was probably the result of GDL reducing pH. The pKa of lactic acid is 3.86, which is the pH at which 50% of lactic acid is dissociated and 50% is undissociated. At the pH ranges in this study, as pH decreased the percentage of undissociated lactic acid should have increased and antimicrobial activity should have increased. Therefore, the reduction in pH caused by GDL increased the antimicrobial activity of SL. The singular application of GDL, EDTA or SL resulted in less than a 0.5 $\log_{10}$ reduction in *E. coli* O157:H7 growth when each additive was applied at the highest level. The differences in microbial growth between singular and combined application of EDTA, SL and GDL revealed that the synergism between these additives increased their overall antimicrobial activity.

The combinations of GDL, EDTA and SL that resulted in less than 10^6 CFU/ml at day 1 were evaluated over seven days of incubation at 35°C. Statistical analysis revealed a significant ($P = 0.04$) treatment by day interaction (Table 3-2). Figure 3-2 presents the growth curves of *E. coli* O157:H7 during incubation. The treatment with 0.5% GDL, 0.372% EDTA and 1.5% SL exhibited a more rapid growth rate than the other treatments. The treatment with 0.5% GDL, 0.745% EDTA and 3.0% SL reduced *E. coli* O157:H7 numbers during incubation. Combinations of EDTA, GDL and SL appeared to extend the lag phase of the growth curve. Partial inhibition by the additives may be the result of limited availability or activity of the additives or *E. coli* O157:H7 overcoming the metabolic stresses associated with the additives. Theoretically, EDTA increases cell membrane permeability in Gram-negative bacteria, thereby enhancing cell membrane penetration by GDL and SL. EDTA's availability and activity may be reduced by binding divalent cations in the growth media instead of on cell membranes. A reduction in EDTA's availability or activity would reduce GDL and SL's penetration into the cell and their ability to inhibit growth. Similarly, a reduction in GDL or SL would reduce the amount of undissociated lactic acid present in the growth medium, which would reduce antimicrobial activity.

For example, the 0.5% GDL, 0.372% EDTA and 1.5% SL treatment combination exhibited the least inhibitory effects on *E. coli* O157:H7. This may have been the result of insufficient EDTA to maximize cell permeability to antimicrobial agents and insufficient SL to provide high enough concentrations of undissociated lactic acid to inhibit growth. Combinations with higher levels of EDTA or SL were more inhibitory toward growth. These trends suggest that EDTA and SL were major contributors to the EDTA, SL and GDL interaction.

Table 3-2. Probability values (P > F) for the effects of selected combinations of glucono-delta-lactone, Nisaplin, disodium ethylenediaminetetraacetate and sodium lactate on *Escherichia coli* O157:H7 growth after extended incubation at 37°C

Source of variation	P > F 0, 0.01 and 0.02 M EDTA[a]	P > F 0, 0.0036 and 0.0072% EDTA
Replication	0.0001	0.2843
Treatment	0.0001	0.0231
Day	0.0001	0.0001
Treatment*Day	0.0403	0.2830

[a]EDTA - disodium ethylenediaminetetraacetate.

Combinations of EDTA, GDL and SL were more effective at inhibiting *E. coli* O157:H7 when SL was at 3.0%. The 0.5% GDL, 0.745% EDTA and 3.0% SL combination exhibited the strongest inhibitory effect on *E. coli* O157:H7, it was almost bactericidal. During the 7 day incubation period, growth was always less than 3 $\log_{10}$ CFU/ml. The effectiveness of this combination indicates that EDTA, GDL and SL were at high enough levels to be available and active throughout incubation. There was a slight increase in *E. coli* O157:H7 counts at day 7, which may be the beginning of logarithmic growth. This may indicate the loss of activity or availability of one or more of the additives.

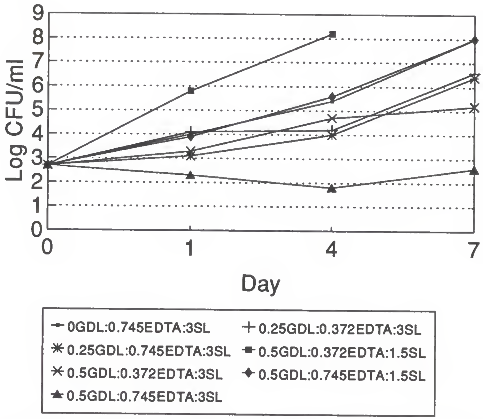

Fig. 3-2—Growth of *Escherichia coli* 0157:H7 in selected combinations of sodium lactate (SL), glucono-delta-lactone (GDL) and disodium ethylenediaminetetraacetate (EDTA) incubated at 37°C for 7 days (STD ERR ± 0.79, n = 4).

EDTA at 0, 0.0036 and 0.0072%

When EDTA was used at 0.372 and 0.745% levels in combination with GDL and SL, there was significant inhibition of *E. coli* O157:H7. However, these levels are 100 times the amount of EDTA allowed in food products. Therefore, EDTA levels were reduced to 0, 0.0036 and 0.0072% disodium EDTA, which are approved for use in food by the FDA. At these levels, significant ($P < 0.0005$) treatment effects were observed for GDL, SL, GDL*SL and GDL*NN*SL (Table 3-1). At low levels of EDTA, there were no significant ($P > 0.05$) interactions between EDTA and GDL, SL or NN. The average counts of *E. coli* O157:H7 for each preservative main effect and preservative combination after 1 day of incubation (37°C) are presented in Appendix A (Tables A-3 and A-4).

Low levels of EDTA did exhibit a main effect ($P = 0.0120$) on growth. Overall treatment averages for combinations containing EDTA revealed that, as EDTA increased from 0 to 0.0072%, numbers declined from 7.4 to 7.3 $\log_{10}$ CFU/ml (Table A-3). Although there were statistical differences, a 0.1 $\log_{10}$ reduction is not of practical significance.

Kraniak and Shelef (1988) reported that 1.7 mM (0.05%) EDTA inhibited the growth of *S. aureus* in casamino acids-yeast extract broth. However, EDTA's antimicrobial activity was lost when Fe^{3+}, Zn^{2+} or Ca^{2+} were incorporated into the broth at 1.6 to 1.8 mM concentrations. When Mg^{2+} was added, EDTA retained its antimicrobial activity until Mg^{2+} concentrations reached 12.3 mM. In this experiment, the concentrations of Fe^{3+}, Zn^{2+} and Ca^{2+} in the CMM may have been equal to the concentration of EDTA used, thus preventing EDTA from binding to Mg^{2+} on *E. coli's* cell membrane. At low levels, EDTA's lack of statistical interactions with GDL and SL and poor inhibitory characteristics may have been the result of metal cations associated with the CMM interfering with EDTA's antimicrobial activity.

Evaluation of the GDL, NN and SL interaction on the growth of *E. coli* O157:H7 revealed that, as GDL and SL levels increased, antimicrobial activity also increased (Fig. 3-3). However, as NN level increased antimicrobial effects of the 0.5% GDL and 3.0% SL treatment groups were reduced. Treatment with NN, 0.5% GDL and 3.0% SL exhibited more growth ($P < 0.0001$) than the treatment with 0.5% GDL and 3.0% SL. The synergistic effects between GDL and SL were likely the result of pH effects on SL. The reduction in pH caused by GDL probably increased the antimicrobial activity of SL. The antagonistic effects of NN with GDL and SL may be related to NN's composition. NN contains denatured milk solids which may function as a nutrient and aid the growth of *E. coli* O157:H7. Singular application of SL, GDL or NN resulted in less than a 0.5 $\log_{10}$ reduction in growth when each additive was applied at the highest level.

Combinations of 0.5% GDL and 3.0% SL with 0, 0.5 and 1% NN were the only treatments exhibiting less than 10^6 CFU/ml of *E. coli* O157:H7 growth after 1 day of incubation. Significant treatment ($P = 0.0231$) and day ($P < 0.0001$) effects occurred during incubation (Table 3-2). Treatment averages were 5.1, 6.5 and 5.8 $\log_{10}$ CFU/ml for 0, 0.5 and 1.0% NN, respectively. Day averages were 2.8, 4.8 and 6.8 $\log_{10}$ CFU/ml at days 0, 1 and 2, respectively (Data not presented in tabular form.).

There was a difference in the *E. coli* O157:H7 numbers at 0% EDTA or 0% NN and 0.5% GDL and 3.0% SL between the first and second experiments. In the first

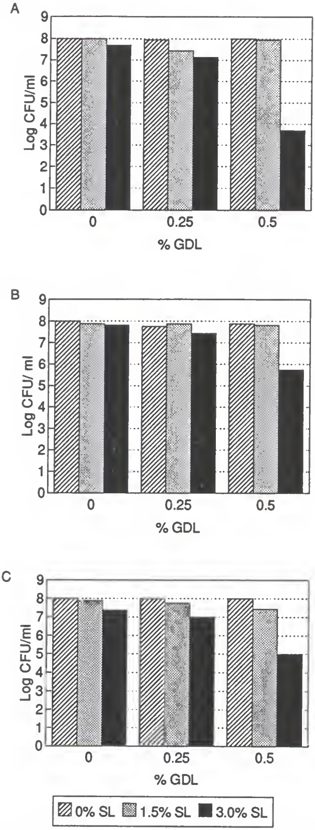

Fig. 3-3-Mean $\log_{10}$ values of *Escherichia coli* 0157:H7 grown in various levels of sodium lactate (SL), glucono-delta-lactone (GDL) and Nisaplin (NN) for 1 day at 37°C (A - 0% NN; B - 0.5% NN; C - 1.0% NN) (STD ERR ± 0.22, n = 4).

experiment, this value is 6.6 $\log_{10}$ CFU/ml. In the second, it is 3.7 $\log_{10}$ CFU/ml. In the second experiment two observations were 2.0 to 3.0 $\log_{10}$ CFU/ml, while the other two observations were 5.0 $\log_{10}$ CFU/ml. The high and low values were equally distributed between both replications.

Conclusions

In the experiment utilizing 0.372 and 0.745% EDTA, NN did not exhibit statistical interactions with EDTA, GDL or SL. Statistically, NN exhibited antimicrobial activity toward *E. coli* O157:H7, however, NN's effects on the reduction in growth was < 0.5 $\log^{10}$ CFU/ml and was not of practical significance. Statistical interactions were observed between EDTA, GDL and SL. The combination of 0.5% GDL, 3.0% sodium lactate and 0.745% EDTA inhibited the growth of *E. coli* O157:H7 for 7 days at 37°C. Unfortunately, the FDA only allows 0.0036% of disodium EDTA in food products which is approximately 200 times less than 0.02 M. The illegality of 0.745% EDTA in cooked sausage products makes its utilization in processed meat products impractical.

At reduced levels of EDTA, there were no statistical interactions between EDTA and GDL, SL or NN. The lack of interactions between EDTA and GDL or SL may have resulted from EDTA interacting with magnesium or other metal cations present in the growth media which would prevent EDTA from binding to magnesium ions on the bacteria's cell membrane. Statistically, low levels of EDTA exhibited antimicrobial activity toward *E. coli* O157:H7. However, the reduction in growth was not practically significant. Statistical interactions were observed between GDL, SL and NN. GDL and

SL exhibited a synergistic interaction, while NN exhibited an antagonistic interaction toward the effects of GDL and SL combinations.

However, these data only address one foodborne pathogen. In order to further evaluate the potential of these additives, they need to be applied to several other foodborne pathogens.

CHAPTER 4

THE EFFECTS OF SODIUM LACTATE, GLUCONO-DELTA-LACTONE, NISAPLIN AND THEIR COMBINATIONS ON THE GROWTH OF SELECTED FOODBORNE PATHOGENS

Introduction

In the previous chapter, the antimicrobial activity of sodium lactate (SL), glucono-delta-lactone (GDL), disodium ethylenediaminetetraacetate (EDTA), Nisaplin (NN) and their combinations toward *Escherichia coli* O157:H7 was investigated. The study was performed with the intent of identifying preservatives or preservative combinations possessing the potential to inhibit the growth of common foodborne pathogens at their optimum temperatures for growth. It was observed that EDTA at 0.372 and 0.745% (0.01 and 0.02 M) exhibited synergistic interactions with GDL and SL to inhibit the growth of *E. coli* O157:H7. However, when EDTA was reduced to legal levels, 0.0036 and 0.0072%, interactions with GDL and SL were lost. Other researchers that have identified EDTA as an antimicrobial agent used it at 5 mg/ml (0.5%), 20 mM (0.75%), 1.5 to 2.5 mg/ml (0.15 to 0.25%) and 8% (Samuelson et al., 1985; Stevens et al., 1991; Payne et al., 1994; Golden et al., 1995). The levels used in these studies all exceeded the levels currently allowed in meat products. Therefore, EDTA was eliminated from this experiment.

In Chapter 3, it was observed that GDL and SL interacted to suppress the growth of *E. coli* O157:H7. Independently, SL has been shown to delay the growth of *Listeria* *monocytogenes*, *Pseudomonas fragi*, *Clostridium sporogenes*, *Salmonella typhimurium* and *E. coli* O157:H7 and the toxin production of *Clostridium botulinum* (Maas et al., 1989; Harmayani et al., 1991; Shelef and Yang, 1991; Weaver and Shelef, 1993; Houtsma et al., 1994; Miller and Acuff, 1994; Wederquist, 1994). Similarly, the independent application of GDL at 1.5% reduced the growth of *L. monocytogenes* in reconstituted milk (El-Shenawy and Marth, 1990). GDL at 0.4 to 1.0%, reduced the growth of *Staphylococcus aureus* in Italian dry sausage (Metaxopolous et al., 1981). In pork liver paté, 0.5% GDL inhibited the growth of lactic acid bacteria (Madden, 1989). The combination of 2.0% SL with 0.25 and 0.5% GDL has been shown to inhibit the growth of *L. monocytogenes* on bologna style sausage at 5 and 10°C for 35 days (Qvist et al., 1994).

In Chapter 3, statistical analysis indicated that Nisaplin, a commercial blend of nisin, reduced the growth of *E. coli* O157:H7 by 0.4 $\log_{10}$ CFU/ml. However, in a literature review Delves-Broughton (1990) stated that nisin does not inhibit Gram-negative bacteria. Nisin does, however, inhibit a wide variety of Gram-positive bacteria, particularly spore formers. Nisin has been shown to inhibit the growth of *C. botulinum*, *L. monocytogenes*, *Listeria ivanovii*, *Listeria innocua*, *S. aureus*, *Streptococcus lactis* and *Bacillus licheniformis* in media and in dairy, fish and meat products (Bell and De Lacy, 1985; Somers and Taylor, 1987; Benkerroum and Sandine, 1988; Taylor et al., 1990; Harris et al., 1991; Motlagh et al., 1991; El-Khateib et al., 1993; Fang and Lin, 1994). Nisin and GDL exhibited synergistic interactions in delaying the outgrowth of *Bacillus* spores (Oscroft et al., 1990)

This research was undertaken to determine the effects of sodium lactate, glucono-delta-lactone, nisaplin and their combinations on the growth of various foodborne pathogens at optimum temperatures for growth.

Materials and Methods

Experimental design

The experimental design was similar to the experiments performed in chapter 3 with the exception that disodium EDTA was not used and different pathogens were investigated. Cooked meat media (CMM) was formulated to contain SL, GDL and NN. A 3 X 3 X 3 factorial design was used with additives at the following levels: SL at 0, 1.5 and 3% (V/V); GDL at 0, 0.25 and 0.5% (W/V); and NN at 0, 0.5 and 1% (W/V), which were 0, 0.0125 and 0.025% nisin, respectively. The treatments were inoculated with *S. typhimurium* ATCC 14028, *Yersinia enterocolitica* ATCC 23715, *L. monocytogenes* ATCC 15313 or *S. aureus* ATCC 12600 to obtain initial levels of 2 to 3.5 $\log_{10}$ CFU/ml. Two replications consisting of duplicate test tubes for each treatment were performed with each organism. The treatments were incubated at 37°C for 3 days with growth being evaluated on days 1, 2 and 3 or until numbers exceeded 10^6. Above 10^6 CFU/ml, treatments were not inhibiting growth and any reductions in numbers would be the result of the organisms entering the death phase of the growth curve.

Cooked meat media preparation

Sodium lactate and GDL were incorporated into the CMM prior to autoclaving (15 min at 121°C). Nisaplin was dissolved in sterile 0.12 N HCl and aseptically added to the sterile CMM. Final volume was 10 ml. Treatment pH varied with the level of GDL in each treatment. The average pH for the GDL levels were 6.0 at 0% GDL and 5.0 to 5.5 at 0.25 and 0.5% GDL. The treatments were heated to 70°C in a water bath and immediately cooled in tap water to simulate cooking of a meat product. The selected pathogens were inoculated into the CMM after it cooled to room temperature.

Inoculum

Each pathogen was inoculated into a specific broth and incubated overnight at 37°C. This resulted in inoculum counts of approximately 10^8. The inoculum was serially diluted to approximately 10^4 in 0.1% peptone water (Difco #1807-17-4). At inoculation, initial levels of each organism were determined by enumerating the treatment combination with no additives. Initial levels ranged from 2 to 3.5 $\log_{10}$ CFU/ml.

The inoculum broths used for each organism were GN broth (Difco #0486-01-2) for *S. typhimurium*, tryptic soy broth (Difco #0370-01-1) for *Y. enterocolitica*, proteose peptone #3 (Difco #0122-17-4) for *L. monocytogenes* and tryptic soy broth for *S. aureus*.

Microbial analysis

Enumeration consisted of serial dilutions through 10^{-6} in 0.1% peptone water and a variation of the drop plate method (Swanson et al., 1992) where two 10 µl drops of each dilution were placed symmetrically around the perimeter of a petri dish. Plates were incubated in the upright position at 37°C for 24 or 48 hours depending upon the organism being investigated. This technique resulted in an enumeration range of 2 to 8 $\log_{10}$ CFU/ml. For statistical analysis, observations with less than 2 $\log_{10}$ CFU/ml of growth were recorded as 1.5 $\log_{10}$ CFU/ml.

The enumeration media for each organism were bismuth sulfite (Difco #0073-01-1) for *S. typhimurium*, *Yersinia* selective (Difco #1817-17-2) for *Y. enterocolitica*, Modified Oxford medium (Difco #0225-17-0) for *L. monocytogenes* and Baird-Parker (Difco #0768-01-1) for *S. aureus*.

Statistical analysis

For day 1 values, the SL, GDL and NN treatments and their combinations as applied to all organisms were statistically analyzed utilizing the general linear models program (PROC GLM) of SAS (SAS Institute, 1985). Variations in microbial growth were divided according to eight treatment effects: replication, GDL, NN, SL, GDL*NN, GDL*SL, NN*SL and GDL*NN*SL. After day 1, treatment combinations that exceeded 6 $\log_{10}$ CFU/ml were eliminated from the experiment which made such a comparison for all days inappropriate. Comparisons between individual means were performed using the lsmean pdiff statement of SAS. Treatment interactions and differences between means were considered significant at $P \leq 0.05$.

Treatments involved in a statistical interaction with less than 10^6 CFU/ml at day one were statistically analyzed to evaluate the inhibitory effects of treatments over time. In order to be selected and evaluated at each day, treatments had to exhibit less than 10^6 CFU/ml in both replications. This resulted in four observations for each treatment at each day. Variations in growth were accounted for by replication, treatment, day of incubation and treatment by day interaction. Differences in the means of each treatment by day combination were identified using the least squared means pdiff statement. Sources of variation and treatment by day differences were considered significant at $P \leq 0.05$.

Results and Discussion

Additive effects and interactions

For one day of incubation, the interaction between GDL, SL and NN was significant ($P < 0.001$) for *L. monocytogenes* and *S. aureus* and was not significant ($P > 0.05$) for *S. typhimurium* or *Y. enterocolitica* (Table 4-1). The interaction of NN with GDL and SL for *S. typhimurium* and *Y. enterocolitica* will not be discussed, as it was not significant. The average counts of *S. typhimurium* and *Y. enterocolitica* in each preservative combination are presented in Appendix A (Tables A-5 and A-6). Significant ($P < 0.0001$) interactions occurred between SL and GDL for all pathogens. Significant ($P < 0.001$) main effects were present in *L. monocytogenes* and *S. aureus* for all of the preservatives. However, NN did not exhibit significant ($P < 0.001$) main effects for *S. typhimurium* or *Y. enterocolitica*. The average levels of microbial growth for each main effect and pathogen are presented in Appendix A (Table A-7). For extended incubation of selected treatment combinations, significant ($P < 0.0001$) treatment by day interactions were observed for *Y. enterocolitica*, *L. monocytogenes* and *S. aureus* as shown in Table 4-2. The effects on one day of incubation and extended incubation are discussed on an organism basis.

Gram-negative pathogens

For *S. typhimurium* at one day of incubation, there was a significant ($P < 0.0001$) interaction between SL and GDL treatment groups (Table 4-1). Figure 4-1 presents the mean $\log_{10}$ values for *S. typhimurium* as a function of GDL and SL levels. At 0.5% GDL, all levels of SL were more inhibitory than they were at lower levels of GDL. The synergistic effects between GDL and sodium lactate are likely the result of pH effects on sodium lactate. The reduction in pH caused by GDL increased the concentration of undissociated lactic acid which resulted in an increase in the antimicrobial activity of sodium lactate. Singularly, sodium lactate caused less than a 0.5 $\log_{10}$ reduction in the growth of *S. typhimurium*. However, a 1 $\log_{10}$ increase in growth was observed with the singular application of GDL.

Table 4-1. Probability values (P > F) for the effects of glucono-delta-lactone, sodium lactate, Nisaplin and their combinations on the growth of selected foodborne pathogens in cooked meat media after 1 day of incubation at 37°C

Source	*S. typhimurium*	*Y. enterocolitica*	*L. monocytogenes*	*S. aureus*
Replication	0.4576	0.3192	0.5752	0.5669
GDL[a]	0.0001	0.0001	0.0001	0.0001
NN[b]	0.0802	0.6656	0.0001	0.0001
SL[c]	0.0001	0.0001	0.0001	0.0001
GDL*NN	0.2977	0.1979	0.0001	0.0001
GDL*SL	0.0001	0.0001	0.0001	0.0001
NN*SL	0.4599	0.5302	0.0001	0.0001
GDL*NN*SL	0.0818	0.9756	0.0001	0.0002

[a] GDL - glucono-delta-lactone.
[b] NN - Nisaplin.
[c] SL - sodium lactate.

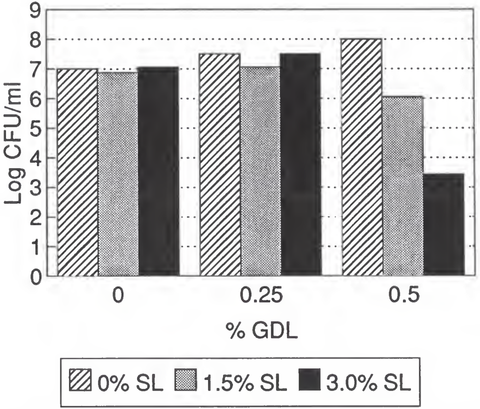

Fig. 4-1—Mean $\log_{10}$ values of *Salmonella typhimurium* grown in various levels of sodium lactate (SL) and glucono-delta-lactone (GDL) for 1 day at 37°C (STD ERR ± 0.37, n = 4).

The 0.5% GDL and 3.0% SL combination was the only treatment combination involved in a statistical interaction that exhibited less than 10^6 CFU/ml of *S. typhimurium* growth after 1 day of incubation. The mean $\log_{10}$ values for this treatment were 2.9, 3.4 and 5.3 CFU/ml at days 0, 1 and 2 respectively (Data not presented in tabular form.).

Table 4-2. Probability values (P > F) for the effects of selected combinations of glucono-delta-lactone, sodium lactate and Nisaplin on the growth of selected foodborne pathogens in cooked meat media for 3 days of incubation at 37°C

Source	*Y. enterocolitica*	*L. monocytogenes*	*S. aureus*
Replication	0.2106	0.0591	0.0401
Treatment	0.0001	0.0001	0.0001
Day	0.0001	0.0001	0.0001
Treatment*Day	0.0001	0.0001	0.0001

There was a significant ($P < 0.0001$) interaction between SL and GDL treatment groups for *Y. enterocolitica* at one day of incubation as shown in Table 4-1. The mean $\log_{10}$ values for *Y. enterocolitica* are presented in Figure 4-2. Increasing the concentration of GDL resulted in increased inhibitory effects. This inhibition was increased when SL was added. No significant differences ($P > 0.05$) in growth were observed between combinations with intermediate and maximum levels of GDL and SL. Singularly, SL exhibited slight reductions in growth. *Y. enterocolitica* is sensitive to acidic conditions (Schiemann, 1989) which may account for GDL's effectiveness in suppressing growth. The enhanced antimicrobial activity of GDL and SL combinations was likely the result of reduced pH increasing the concentration of undissociated lactic acid.

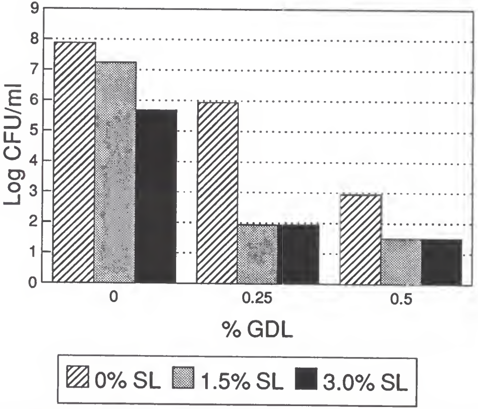

Fig. 4-2—Mean $\log_{10}$ values of *Yersinia enterocolitica* grown in various levels of sodium lactate (SL) and glucono-delta-lactone (GDL) for 1 day at 37°C (STD ERR ± 0.39, n = 4).

The mean $\log_{10}$ values of selected GDL and SL treatment combinations after extended incubation are presented in Figure 4-3. These treatment combinations were involved in a statistical interaction after one day of incubation and exhibited less than 10^6 CFU/ml of growth in both replications. Statistical analysis revealed treatment by day interactions ($P < 0.0001$) as presented in Table 4-2. The statistical interaction appeared to be the result of differences in growth rates between treatments. After incubation for 3 days, the 0.5% GDL-0% SL and 0.25% GDL-1.5% SL combinations exhibited 7 $\log_{10}$ CFU/ml of growth while the 0.25% GDL-3.0% SL, 0.5% GDL-1.5% SL and 0.5% GDL-3.0% SL combinations exhibited ≥ 3 $\log_{10}$ CFU/ml of growth. The 0.5% GDL-3.0% SL appeared to be bactericidal with *Yersinia* levels being less than 2 $\log_{10}$ CFU/ml throughout incubation. Overall, combinations of GDL and SL appeared to increase the length of the lag phase. Increasing concentrations of both GDL and SL resulted in extensions of the observed lag phase.

Y. enterocolitica appeared to be more sensitive to treatment with GDL and SL than *S. typhimurium*. This trend might be associated with *Yersinia* species being more sensitive to acidic pHs than *S. typhimurium*.

Nisaplin and its interactions with GDL or SL did not reduce *S. typhimurium* or *Y. enterocolitica* growth. This agrees with Chung et al. (1989), Delves-Broughton (1990), Motlagh et al. (1991) and Stevens et al. (1991) who reported that nisin is ineffective in reducing the growth of Gram-negative bacteria.

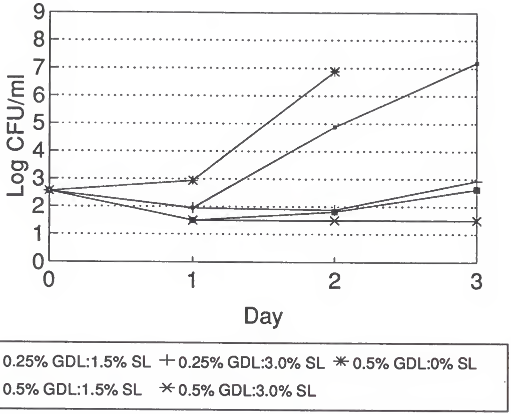

Fig. 4-3—Growth of *Yersinia enterocolitica* in selected combinations of sodium lactate (SL) and glucono-delta-lactone (GDL) incubated at 37°C for 3 days (STD ERR ± 0.46, n = 4).

Gram-positive pathogens

For *L. monocytogenes* at one day of incubation, the interaction of NN, GDL and SL treatment groups was significant ($P < 0.0001$) as shown in Table 4-1. The mean $\log_{10}$ values for *L. monocytogenes* as a function of NN, GDL and SL are presented in Figure 4-4. After 1 day of incubation, all combinations of NN, GDL and SL exhibited less than 2 $\log_{10}$ CFU/ml of *L. monocytogenes*, indicating that these combinations were either inhibitory or bactericidal. The singular use of Nisaplin at 0.5 and 1.0% also resulted in less than 2 $\log_{10}$ CFU/ml of *L. monocytogenes* (Fig. 4-4 B). Nisaplin's inhibitory affect was observed throughout the 3 day incubation, indicating that Nisaplin is bactericidal to *L. monocytogenes* (Fig. 4-5). These data indicated that NN may be responsible for the strong inhibitory affects of the NN, GDL and SL interaction. Nisin's inhibitory activity toward *L. monocytogenes* has been reported by Benkerroum and Sandine (1988), Chung et al. (1989), Delves-Broughton (1990) and Harris et al. (1991).

Significant ($P < 0.0001$) interactions were also observed between GDL and SL treatment groups for *L. monocytogenes* at one day of incubation (Table 4-1). The mean $\log_{10}$ values for *L. monocytogenes* as a function of GDL and SL concentrations are presented in Figure 4-4A. When both GDL and SL concentrations were increased, there was a corresponding increase in antimicrobial activity. Combinations of 0.25% GDL and 3.0% SL, 0.5% GDL and 1.5% SL, and 0.5% GDL and 3.0% SL resulted in 2 or less than 2 $\log_{10}$ CFU/ml of growth. The 0.5% GDL and 1.5% SL, and 0.5% GDL and 3.0% SL treatment combinations were not significantly different ($P > 0.05$) from the NN treatments. Qvist et al. (1994) also reported a synergistic inhibition of *L. monocytogenes*

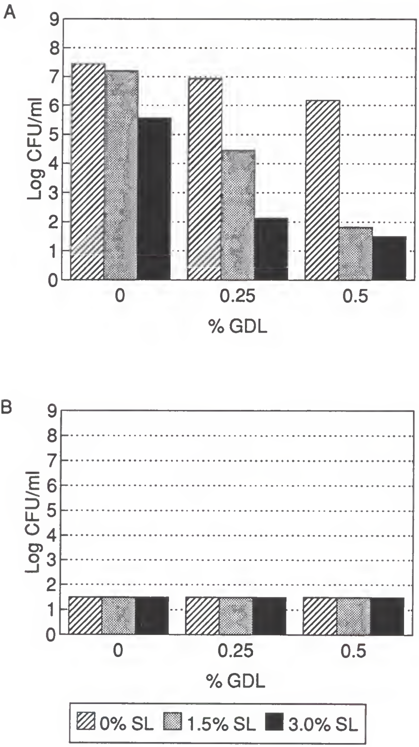

Figure 4-4—Mean $\log_{10}$ values of *Listeria monocytogenes* grown in various levels of sodium lactate (SL), glucono-delta-lactone (GDL) and Nisaplin (NN) for 1 day at 37°C (A - 0% NN; B - 0.5% and 1.0% NN) (STD ERR ± 0.20, n = 4).

by SL and GDL in bologna type sausages. They observed that 2% SL in combination with 0.25 and 0.5% GDL prevented growth for 35 days at 5 and 10°C. Singularly, 2% SL suppressed growth for 28 days at 5°C, but did not suppress growth at 10°C.

The growth of *L. monocytogenes* was reduced by the singular application of SL and GDL. Independently, 3.0% SL reduced growth by 2 $\log_{10}$ CFU/ml, when compared to the no additive treatment. Sodium lactate's ability to inhibit *L. monocytogenes* in refrigerated meat has been documented. Weaver and Shelef (1993) observed that 3 and 4% SL reduced tthe growth of *L. monocytogenes* in pork liver sausage held at 5°C for 50 days. Wederquist et al. (1994) reported that 2.0% SL reduced the growth of *L. monocytogenes* on vacuum packaged turkey bologna held at 4°C. In this study, 0.5% GDL reduced growth by 1 $\log_{10}$ value. Similarly, El-Shenawy and Marth (1990) reported that 0.3% GDL in tryptose broth and 0.5% GDL in reconstituted milk reduced the growth of *L. monocytogenes*.

The mean $\log_{10}$ values of *L. monocytogenes* in selected NN, GDL and SL treatment combinations after extended incubation are presented in Figure 4-5. These combinations were involved in a statistical interaction after one day of incubation and exhibited less than 10^6 CFU/ml of growth in both replications. All treatment combinations containing NN exhibited less than 10^2 CFU/ml of growth throughout the 3 day incubation and were recorded as 1.5 $\log_{10}$ CFU/ml. These data were equal and were therefore combined and presented as Nisaplin in Figure 4-5. However, the NN treatment combinations were statistically analyzed as individual treatments. Statistical analysis revealed a significant ($P < 0.0001$) treatment by day interaction as shown in Table 4-2.

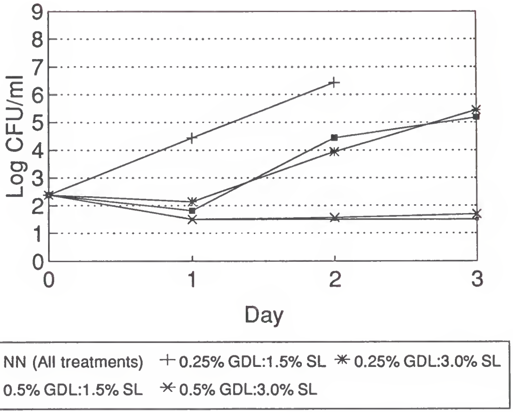

Fig. 4-5—Growth of *Listeria monocytogenes* in selected combinations of sodium lactate (SL), glucono-delta-lactone (GDL) and Nisaplin (NN) incubated at 37°C for 3 days (STD ERR ± 0.22, n = 4).

The statistical interactions appeared to be the result of rapid growth in the 0.25% GDL and 3.0% SL combination and complete inhibition by the 0.5% GDL and 3.0% SL combination and the combinations containing NN. The 0.5% GDL and 3.0% SL combination was as effective as NN at inhibiting the growth of *L. monocytogenes*.

There was a significant ($P = 0.0002$) interaction between NN, GDL and SL treatment groups for *S. aureus* at one day of incubation as shown in Table 4-1. The mean $\log_{10}$ values as a function of NN, GDL and SL are presented in Figure 4-6. Growth was less than 10^2 CFU/ml when NN, GDL and SL were present. However, in the absence of SL there appeared to be an antagonistic interaction between NN and GDL. Statistical analysis identified significant ($P < 0.0001$) two way interactions between NN and SL, NN and GDL and SL and GDL (Table 4-1). The NN and SL interaction appeared to be the result of NN's inhibitory activity. Singularly, 0.5 and 1.0% NN suppressed growth to less than 2 $\log_{10}$ CFU/ml. The NN treatments were not significantly ($P > 0.05$) different from the 0.5% GDL, 3.0% SL and 1.0% NN treatment combination. At 0% GDL, including NN within any SL level reduced growth by 5 or more $\log_{10}$ cycles. However, including SL within any NN level, at 0% GDL resulted in less than a 1 $\log_{10}$ reduction in *S. aureus* numbers. Nisin's inhibitory activity toward *S. aureus* has been documented by Chung et al. (1989) and Delves-Broughton (1990).

The NN and GDL interaction appeared to be antagonistic toward inhibiting the growth of *S. aureus*. Combinations of GDL and NN exhibited more ($P < 0.05$) growth than NN alone. This suggests that *S. aureus'* response to acidic pH or GDL may affect its response to Nisaplin. Thomas and Wimpenny (1996) observed a similar reaction at

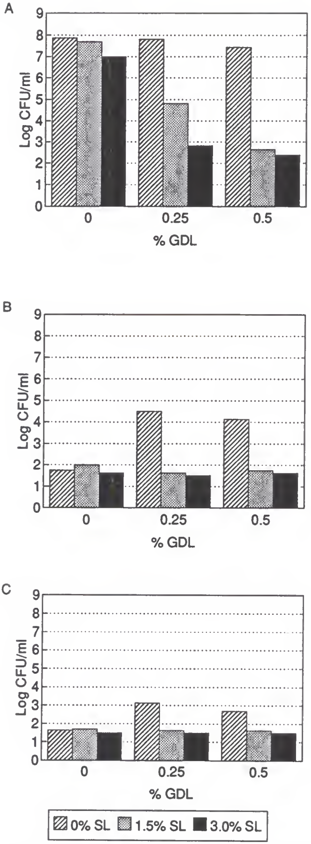

Fig. 4-6—Mean $\log_{10}$ values of *Staphylococcus aureus* grown in various levels of sodium lactate (SL), glucono-delta-lactone (GDL) and Nisaplin (NN) for 1 day at 37°C (A - 0% NN; B - 0.5% NN; C - 1.0% NN) (STD ERR ± 0.35, n = 4).

pH 4.5-5.0 and 20-25°C where *S. aureus* exhibited increased resistance to combinations of nisin and sodium chloride.

The GDL and SL interaction appeared to be synergistic. The mean $\log_{10}$ values of *S. aureus* as a function of GDL and SL are presented in Figure 4-6A. As GDL and SL levels increased, antimicrobial activity also increased. The combinations of 0.25% GDL and 3.0% SL, 0.5% GDL and 1.5% SL, 0.5% GDL and 3.0% SL reduced growth by 5.0 to 5.5 $\log_{10}$ cycles and were not significantly different ($P > 0.05$) from each other. The independent application of GDL and SL reduced growth by less than 1 $\log_{10}$ cycle.

The mean $\log_{10}$ values of *S. aureus* in selected NN, GDL and SL treatment combinations after extended incubation are presented in Figure 4-7. These treatment combinations were involved in a statistical interaction and exhibited less than 10^6 CFU/ml after one day of incubation. Statistical analysis indicated a significant ($P < 0.0001$) treatment by day interaction as shown in Table 4-2. The statistical interaction appeared to be the result of different growth rates between treatment combinations. The 0.25% GDL and 1.5% SL combination exhibited rapid growth for the first two days of incubation, while the other treatments inhibited growth for the first day of incubation. Singularly, 1% NN was more effective at reducing the growth rate of *S. aureus* than NN and SL combinations and most of the NN, GDL and SL combinations. The 1.0% NN, 3.0% SL and 0.5% GDL combination inhibited growth for 3 days and appeared to be bactericidal.

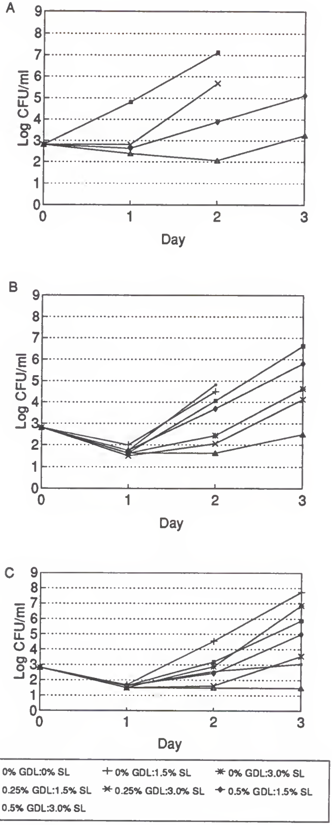

Fig. 4-7—Growth of *Staphylococcus aureus* in selected combinations of sodium lactate (SL), glucono-delta-lactone (GDL) and Nisaplin (NN) incubated at 37°C for 3 days (A - 0% NN; B - 0.5% NN; C - 1.0% NN) (STD ERR ± 0.61, n = 4).

Nisaplin inhibited the growth of *L. monocytogenes* and *S. aureus*. These observations are supported by Delves-Broughton (1990) who reports that nisin inhibits a wide variety of Gram-positive bacteria.

Glucono-delta-lactone and SL exhibited synergistic interactions at inhibiting the growth of *L. monocytogenes* and *S. aureus*. A similar interaction was observed between gluconic acid and lactic acid when they were used in combination as a fresh beef decontaminant (Zepeda et al., 1994). Refrigerated, vacuum packaged *semimembranosus* samples treated with the combination of 1.5% lactic acid and 3.0% gluconic acid exhibited approximately 0.8 and 1.5 $\log_{10}$ CFU/cm^2 less aerobic psychrotrophic and lactic acid bacteria growth, respectively, than samples treated with either 1.5% lactic acid or 3.0% gluconic acid.

The synergistic antimicrobial activity of SL and GDL may be the result of several mechanisms 1) reduced pH, 2) increased lactate and gluconic acid ion concentration or 3) a chemical interaction between lactate and gluconic acid that results in the increased antimicrobial activity of one or both ions. In general, reducing the pH of the growth medium will result in an increased percentage of undissociated acid. The undissociated form of an acid is responsible for its antimicrobial activity. Therefore, as the percentage of undissociated lactic acid or gluconic acid in the growth medium increases the antimicrobial activity of the acids also increases. The concentration of lactate has been observed to be the principal antibotulinal factor when sodium lactate and sodium chloride were incorporated into reinforced clostridial media (Maas et al., 1989). High extracellular concentrations of lactate probably inhibited lactate efflux from the cell, reducing ATP generation from proton transfer across the cell membrane. The combination of both lactate and gluconic acid ions in high extracellular concentrations may enhance the inhibition of lactate efflux, further reducing ATP generation, thus having a greater impact on cell growth and survival. Finally differences in the pKas of lactic acid (3.86) and gluconic acid (3.70) may result in gluconic acid donating protons to lactic acid (lactate ions), thus increasing the concentration of undissociated lactic acid which would increase lactic acid's antimicrobial activity.

Conclusions

Nisaplin was effective at inhibiting the growth of the Gram-positive organisms tested. However, it was ineffective at inhibiting the Gram-negative organisms evaluated. Nisaplin did not exhibit synergistic activity with SL or GDL for preventing the growth of *S. typhimurium* or *Y. enterocolitica*. Nisaplin exhibited an antagonistic interaction with GDL when applied to *S. aureus*. The combination of NN's narrow range of antimicrobial activity and its antagonistic interaction with GDL will limit its use in meat and food products containing meat.

Combinations of GDL and SL were effective at suppressing the growth of Gram-positive and Gram-negative pathogens at optimum growth temperatures. Their antimicrobial activity indicates that combinations of GDL and SL may be useful for preserving processed meat and other food products. In combination, GDL and SL appear to have the potential to be used to reduce the growth of foodborne pathogens at their optimum growth temperatures. The mechanism of the GDL and SL interaction is thought to be the result of reduced pH, the concentration of lactate and gluconic acid ions and/or gluconic acid increasing the concentration of undissociated lactic acid. This theory will be discussed and investigated in Chapter 5.

CHAPTER 5

THE EFFECTS OF SODIUM LACTATE, GLUCONO-DELTA-LACTONE AND DISODIUM EDTA ON THE GROWTH OF *ESCHERICHIA COLI* O157:H7 AND *SALMONELLA TYPHIMURIUM* IN COOKED MEAT MEDIA

Introduction

In Chapters 3 and 4, the antimicrobial activity of sodium lactate (SL), glucono-delta-lactone (GDL), Nisaplin (NN, a commercial blend of nisin), disodium ethylenediaminetetraacetate (EDTA) and their combinations toward *Escherichia coli* O157:H7, *Salmonella typhimurium*, *Yersinia enterocolitica*, *Listeria monocytogenes* and *Staphylococcus aureus* was investigated. These studies were performed with the intent of identifying a preservative or preservative combination that possess the potential to inhibit the growth of common foodborne pathogens at their optimum temperatures for growth. Nisaplin only exhibited antimicrobial activity toward the Gram-positive organisms tested. There was also an antagonistic interaction between GDL and NN which suppressed NN antimicrobial activity toward *S. aureus*. Similarly, there was a slight antagonistic interaction between SL, GDL and NN that enhanced the growth of *E. coli* O157:H7 when NN was used with combinations of GDL and SL. Therefore, NN was not utilized in this experiment.

In Chapters 3 and 4, GDL and SL exhibited a synergistic antimicrobial interaction toward all five of the organisms evaluated. Independently, SL has been shown to delay the growth of *L. monocytogenes*, *Pseudomonas fragi*, *S. typhimurium*, *Clostridium* *sporogenes* and *E. coli* O157:H7 and the toxin production of *Clostridium botulinum* (Maas et al., 1989; Harmayani et al., 1991; Shelef and Yang, 1991;. Weaver and Shelef, 1993; Houtsma et al., 1994; Miller and Acuff, 1994; Wederquist, 1994). The USFDA recognizes SL as a GRAS ingredient for direct use in human foods, with the exception of infant foods and formula (USFDA, HHS, 1995). Similarly, GDL is commonly used in some fermented sausages at 0.5% and is allowed in Genoa salami at 1.0% (Romans et al., 1977). El-Shenawy and Marth (1990) observed that 1.5% GDL reduced the growth of *L. monocytogenes* in reconstituted milk at 13°C. At 0.4 to 1.0%, GDL reduced the growth of *S. aureus* in Italian dry sausage (Metaxopolous et al., 1981). In pork liver pate', 0.5% GDL inhibited the growth of lactic acid bacteria (Madden, 1989). The combination of 2.0% SL with 0.25 and 0.5% GDL has been shown to inhibit the growth of *L. monocytogenes* on bologna style sausage at 5 and 10°C for 35 days (Qvist et al., 1994).

In Chapter 3, 0.372 and 0.745% EDTA exhibited synergistic interactions with GDL and SL to inhibit the growth of *E. coli* O157:H7 in cooked meat media. However, when the EDTA concentration was reduced to 0.0072% its interactions with GDL and SL were lost. EDTA is a metal chelating agent that functions as both an antioxidant and antimicrobial agent. The antioxidant activity involves binding divalent metal cations which prevents them from oxidizing lipids. EDTA has been shown to inhibit the growth of *S. typhimurium* and *Salmonella heidelberg* in trypticase soy broth at 0.5% (Samuelson et al., 1985) and *S. aureus* strain 196E in BHI broth at 0.8 to 1.7 mM or 0.024 to 0.05% (Kraniak and Shelef, 1988). EDTA exhibits synergistic antimicrobial effects with nisin

(Stevens et al., 1991; Cutter and Siragusa, 1995a, 1995b), lysozyme (Teotia and Miller, 1975; Payne et al., 1994), ascorbic acid, sodium acetate and sodium propionate (Golden et al., 1995) and sodium nitrite (Tompkin et al., 1978, 1979). Disodium EDTA is allowed in cooked sausages at levels of 0.0036% and pie fillings at 0.05% (USFDA, HHS, 1995).

This research was undertaken to determine the effects of sodium lactate, glucono-delta-lactone, disodium EDTA and their combinations on the growth of *E. coli* O157:H7 and *S. typhimurium* at optimum temperatures for growth. The potential modes of action for the synergistic interactions of SL, GDL and EDTA were also evaluated.

Materials and Methods

Experimental design

Cooked meat media (Difco Laboratories, Detroit, MI, #0267-01-7) were formulated to contain SL (natural 60% USP liquid, Purac, Inc., Arlington Heights, IL), GDL (Sigma Chemical Co., St Louis, MO) and disodium EDTA (Fisher Scientific, Fair Lawn, NJ). The experiments utilized a 3 X 3 X 2 factorial design with additives at the following levels: SL at 0, 1.5 and 3% (V/V); GDL at 0, 0.25 and 0.5% (W/V); and EDTA at 0 and 0.05% (W/V, 0 and 500 ppm). Treatments were inoculated with *E. coli* O157:H7 ATCC 43895 or *S. typhimurium* ATCC 13311 to obtain initial levels of 4 to 5 $\log_{10}$ CFU/ml. Three replications consisting of triplicate test tubes for each treatment were performed for each organism. The treatments were incubated at 37°C for 3 days with growth and pH being evaluated at days 1, 2 and 3. If numbers were greater than 10^6 CFU/ml after day 1, the microbial and pH analysis were discontinued.

Cooked meat media preparation

A 400 ml volume of cooked meat media (CMM) was prepared and buffered with a combination of 0.1 M NaH_2PO_4 (monobasic) and 0.1 M Na_2HPO_4 (dibasic) as described by Costilow (1981) to yield an initial pH of 6.0. The CMM was filtered through cheese cloth to separate the liquid and pellet portions. Treatment tubes were prepared with approximately 1.5 grams of pellets and 6.5 ml of liquid. The CMM was autoclaved (15 min at 121°C). Solutions of 30% sodium lactate, 60% sodium lactate, 5% GDL, 10% GDL and 1% EDTA were filter sterilized. Treatment combinations were prepared by incorporating 0.5 ml of the appropriate additive solution or autoclaved distilled deionized water to the CMM tubes. The CMM treatments were then heated to 70°C in a water bath to simulate cooking of a meat product and the effects of cooking on the various additives. *E. coli* O157:H7 or *S. typhimurium* were inoculated (0.5 ml) into the CMM after it cooled to room temperature.

Inoculum

Each pathogen was inoculated into a specific broth and incubated for 18 hr at 37°C. The resulting inoculum counts were 10^8 to 10^9 CFU/ml. The inoculum was serially diluted to approximately 10^6 CFU/ml in 0.1% peptone water (Difco #1807-17-4). At inoculation, initial levels of each organism were determined by enumerating the treatment combination with no additives. Initial levels were 4 to 5 $\log_{10}$ CFU/ml. The inoculum broths for each organism were tryptic soy broth (Difco #0370-01-1) for *E. coli* O157:H7 and GN broth (Difco #0486-01-2) for *S. typhimurium*.

Microbial analysis

Enumeration consisted of serial dilutions in 0.1% peptone water to 10^{-6} and a variation of the drop plate method (Swanson et al., 1992) where two 10µl drops of each dilution were placed symmetrically around the perimeter of duplicate petri dishes. Plates were incubated in the upright position at 37°C for 24 to 48 hr. The enumeration range of this technique was 2 to 8 $\log_{10}$ CFU/ml. The counts from each pair of duplicate petri dishes were averaged to yield one of three observations for each treatment within a replication. For statistical analysis, observations with less than 2 $\log_{10}$ CFU/ml of growth were recorded as 1.5 $\log_{10}$ CFU/ml. *E. coli* O157:H7 and *S. typhimuriium* were enumerated with EMB Levine (Difco #0005-01-4) and bismuth sulfite (Difco #0073-17-3), respectively.

pH analysis

The pH was analyzed in all treatments before and after the simulated cooking stage of the media preparation and at days 1, 2 and 3 of incubation. The pH analysis was discontinued if a treatment exceeded 6 $\log_{10}$ CFU/ml of growth at day 1. Analysis was performed with a portable Oyster (Extech Instruments Corporation, Waltham, MA) pH meter.

Statistical analysis

For day 1 of the SL, GDL and EDTA combinations the statistical analysis utilized the general linear models program (PROC GLM) of SAS (SAS Institute, 1985). Variations in microbial growth were divided according to eight treatment effects: replication, GDL, EDTA, SL, GDL*EDTA, GDL*SL, EDTA*SL and GDL*EDTA*SL.

After day 1, treatment combinations that exceeded 6 $\log_{10}$ CFU/ml were eliminated from the experiment making such a comparison for all days inappropriate. Comparisons between individual means were performed using the lsmean pdiff statement of SAS. Treatment effects and differences were considered significant when $P > F$ was ≤ 0.05.

Treatments with microbial growth of 10^6 or less CFU/ml at day 1 were statistically analyzed to evaluate their inhibitory effects over time. In order to be selected and evaluated after day 1, treatments had to average 6 $\log_{10}$ CFU/ml or less for all replications. This resulted in nine observations for each treatment at each day. Variations in growth were accounted for by replication, treatment, day of incubation, and treatment by day interaction. Treatment effects were considered significant when $P > F$ was ≤ 0.05.

Results and Discussion

Treatment effects on pH before inoculation

The probability values ($P > F$) obtained from the analysis of variance of pH at day 0 before and after cooking to 70°C indicate a significant ($P = 0.0001$) GDL by SL interaction for experiments inoculated with *E. coli* O157:H7 and *S. typhimurium* (Table 5-1). The pHs of CMM before inoculation with *E. coli* O157:H7 and *S. typhimurium* are presented in Tables 5-2 and 5-3, respectively. When GDL concentration was increased from 0 to 0.5% in any combination of SL and EDTA there was a corresponding 1.3 to 1.6 unit decrease in pH. Similarly, when SL concentration was increased from 0 to 3.0% in the presence of 0.25 and 0.5% GDL there was a corresponding 0.1 to 0.2 unit increase in the CMM pH. This trend of increasing pH in GDL treatments in the presence of SL characterizes the GDL by SL interaction. This interaction was likely the effect of the pHs of the solutions used to prepare the various treatment combinations. The solutions and their pHs were 30% sodium lactate, 6.87; 60% sodium lactate, 7.53; 5% GDL, 2.32; 10% GDL, 2.21; 1% EDTA, 4.50; and distilled deionized water, 5.71. When SL and GDL were used in combination, the low pH of the GDL solutions appeared to be counteracted by the comparatively high pH of the SL solutions. However, when used alone SL did not elevate CMM pH.

Prior to inoculation with either *E. coli* O157:H7 or *S. typhimurium* there were slight differences between the average pH of treatments for each organism. Replication was a source of variation ($P = 0.0001$) within the experiments for each organism. The difference in average pH for each treatment between the experiments for each organism was generally ≤ 0.01 units and was probably caused by slight differences in the media preparation between replications.

Table 5-1. Probability values (P > F) for the effects of glucono-delta-lactone, sodium lactate, disodium ethylenediaminetetraacetate and their combinations on the pH of the media before and after cooking at day 0 prior to inoculation with *Escherichia coli* O157:H7 and *Salmonella typhimurium* in cooked meat media

Source	*E. coli* O157:H7		*S. typhimurium*	
	pH before cook	pH after cook	pH before cook	pH after cook
Replication	0.0001	0.0015	0.0001	0.0015
GDL[a]	0.0001	0.0001	0.0001	0.0001
SL[b]	0.0001	0.0001	0.0001	0.0001
EDTA[c]	0.0001	0.0075	0.0001	0.0001
GDL*EDTA	0.2013	0.6493	0.4735	0.8529
GDL*SL	0.0001	0.0001	0.0001	0.0001
SL*EDTA	0.8580	0.3481	0.1132	0.0363
GDL*SL*EDTA	0.9761	0.9745	0.2027	0.1883

[a] GDL - glucono-delta-lactone.

[b] SL - sodium lactate.

[c] EDTA - disodium ethylenediaminetetraacetate.

Table 5-2. The pH of cooked meat media containing combinations of glucono-delta-lactone, sodium lactate and disodium ethylenediaminetetraacetate before and after simulated cooking to 70°C prior to inoculation with *Escherichia coli* O157:H7 (STD ERR ± 0.011)

EDTA[c] (%)	SL[b] (%)	GDL[a] (%)		
		0	0.25	0.5
Before simulated cook				
0	0	6.06	5.80	5.48
0	1.5	6.06	5.89	5.70
0	3.0	6.06	5.88	5.73
0.05	0	6.04	5.76	5.44
0.05	1.5	6.04	5.85	5.66
0.05	3.0	6.03	5.84	5.69
After simulated cook				
0	0	6.06	5.78	5.47
0	1.5	6.04	5.85	5.65
0	3.0	6.04	5.85	5.67
0.05	0	6.05	5.75	5.47
0.05	1.5	6.03	5.84	5.64
0.05	3.0	6.02	5.82	5.65

[a] GDL - glucono-delta-lactone.
[b] SL - sodium lactate.
[c] EDTA - disodium ethylenediaminetetraacetate.

Table 5-3. The pH of cooked meat media containing combinations of glucono-delta-lactone, sodium lactate and disodium ethylenediaminetetraacetate before and after simulated cooking to 70°C prior to inoculation with *Salmonella typhimurium* (STD ERR ± 0.019)

EDTA[c] (%)	SL[b] (%)	GDL[a] (%)		
		0	0.25	0.5
Before simulated cook				
0	0	6.11	5.74	5.37
0	1.5	6.09	5.85	5.71
0	3.0	6.06	5.86	5.63
0.05	0	6.08	5.67	5.26
0.05	1.5	6.06	5.84	5.65
0.05	3.0	6.05	5.80	5.63
After simulated cook				
0	0	6.15	5.75	5.39
0	1.5	6.11	5.89	5.72
0	3.0	6.11	5.86	5.60
0.05	0	6.09	5.68	5.29
0.05	1.5	6.08	5.87	5.65
0.05	3.0	6.07	5.83	5.62

[a] GDL - glucono-delta-lactone.
[b] SL - sodium lactate.
[c] EDTA - disodium ethylenediaminetetraacetate.

Treatment effects on pH and microbial growth during incubation

The statistical analysis of the pH of the CMM and growth of *E. coli* O157:H7 after 1 day of incubation indicated a significant GDL*SL*EDTA interaction for pH (P = 0.0003) and growth (P = 0.0001) as seen in Table 5-4. The average pHs of the treatment combinations inoculated with *E. coli* O157:H7 are presented in Table 5-5. Increasing GDL and EDTA concentrations generally reduced pH, while increasing the SL concentration increased the pH. The strongest interaction between additives appeared to be between SL and GDL. When 1.5 or 3.0% SL was combined with 0.25 or 0.5% GDL the pH was 0.18 to 2.6 units higher than treatments containing only 0.25% or 0.5% GDL and 0.17 to 0.36 units lower than treatments containing only 1.5 or 3.0% SL. When EDTA was combined with either GDL or SL there was a 0.02 to 0.09 unit reduction in pH.

Table 5-4. Probability values (P > F) for the effects of glucono-delta-lactone, sodium lactate, disodium ethylenediaminetetraacetate and their combinations on the pH of the media and the growth of *Escherichia coli* O157:H7 and *Salmonella typhimurium* in cooked meat media after 1 day of incubation at 37°C

Source	*E. coli* O157:H7		*S. typhimurium*	
	pH	CFU	pH	CFU
Replication	0.0009	0.0030	0.0001	0.0127
GDL[a]	0.0001	0.0001	0.0001	0.0001
SL[b]	0.0001	0.0001	0.0001	0.0001
EDTA[c]	0.0001	0.0001	0.0001	0.0001
GDL*EDTA	0.0214	0.0001	0.0133	0.2173
GDL*SL	0.0001	0.0001	0.0001	0.0001
SL*EDTA	0.0001	0.0001	0.0117	0.0024
GDL*SL*EDTA	0.0003	0.0001	0.6342	0.2752

[a] GDL - glucono-delta-lactone.
[b] SL - sodium lactate.
[c] EDTA - disodium ethylenediaminetetraacetate.

Table 5-5. The pH of cooked meat media containing combinations of glucono-delta-lactone, sodium lactate and disodium ethylenediaminetetraacetate and inoculated with *Escherichia coli* O157:H7 after 1 day of incubation at 37°C (STD ERR ± 0.006)

EDTA[c] (%)	SL[b] (%)	GDL[a] (%)		
		0	0.25	0.5
0	0	6.09	5.74	5.56
0	1.5	6.09	5.92	5.73
0	3.0	6.10	5.92	5.76
0.05	0	6.02	5.65	5.52
0.05	1.5	6.07	5.90	5.73
0.05	3.0	6.08	5.91	5.73

[a] GDL - glucono-delta-lactone.
[b] SL - sodium lactate.
[c] EDTA - disodium ethylenediaminetetraacetate.

The average levels ($\log_{10}$ CFU/ml) of *E. coli* 0157:H7 after 1 day of incubation are presented in Table 5-6. The three-way GDL*SL*EDTA interaction identified in Table 5-4 can be observed by comparing the levels of *E. coli* O157:H7 in GDL and SL combinations without EDTA with their corresponding combinations with EDTA. The levels of *E. coli* generally decreased as the concentrations of GDL and SL increased. Differences in growth between GDL and SL combinations with and without EDTA ranged between 0 to 2.5 $\log_{10}$ CFU/ml. Treatments containing EDTA were less than or equal to those without EDTA. The greatest difference in growth caused by EDTA appeared at 0% GDL and 3.0% SL. At 1.5% SL and all levels of GDL there was a 1 to 2.1 $\log_{10}$ reduction in growth when EDTA was included. The GDL*SL*EDTA interaction appears to have resulted from strong GDL*SL and EDTA*SL interactions. When EDTA was at 0% the 0.5% GDL-3.0% SL treatment exhibited 3.9 $\log_{10}$ CFU/ml less growth than the control (0% GDL-0% SL-0% EDTA). Similarly, when GDL was at 0% the 0.05% EDTA-3.0% SL treatment exhibited 3.7 $\log_{10}$ CFU/ml less growth than the control. However, at 0% SL the 0.5% GDL-0.05% EDTA treatment only exhibited 0.7 $\log_{10}$ CFU/ml less growth than the control, indicating that the GDL*EDTA interaction was less effective than the GDL*SL and SL*EDTA interactions.

Table 5-6. Levels ($\log_{10}$ CFU/ml) of *Escherichia coli* O157:H7 in cooked meat media containing combinations of glucono-delta-lactone, sodium lactate and disodium ethylenediaminetetraacetate after 1 day of incubation at 37°C (STD ERR ± 0.11)

EDTA[c] (%)	SL[b] (%)	GDL[a] (%)		
		0	0.25	0.5
0	0	8.0	7.8	7.6
0	1.5	7.3	7.1	5.8
0	3.0	6.8	4.7	4.1
0.05	0	7.5	7.2	7.3
0.05	1.5	6.0	5.0	4.8
0.05	3.0	4.3	4.2	4.1

[a] GDL - glucono-delta-lactone.

[b] SL - sodium lactate.

[c] EDTA - disodium ethylenediaminetetraacetate.

The results of the statistical analysis for CMM pH and *S. typhimurium* growth after 1 day of incubation (37°C) are presented in Table 5-4. The pH was significantly affected by GDL*EDTA (P = 0.0133), SL*EDTA (P = 0.0117) and GDL*SL (P = 0.0001) interactions. The average pHs of the treatment combinations are shown in Table 5-7. When EDTA was combined with the treatments containing 0.5% GDL-0% SL and 0% GDL-3.0% SL there were corresponding pH reductions of 0.11 and 0.01, respectively. These reductions are relatively small and correspond with the GDL*EDTA and SL*EDTA interactions. These interactions although statistically significant do not appear to be practically significant. The pH for the 0.5% GDL-3.0% SL treatment combination was 0.36 pH units higher than the pH of the 0.5% GDL-0% SL treatment and 0.40 pH units lower than the 0% GDL-3.0% SL treatment. Intermediate combinations of GDL and SL exhibited similar trends with the pH being 0.2 to 0.4 pH units higher than the treatments without SL and 0.20 to 0.30 units lower than the treatments without GDL. Therefore the GDL*SL interaction was considered to be practically significant.

Table 5-7. The pH of cooked meat media containing combinations of glucono-delta-lactone, sodium lactate and disodium ethylenediaminetetraacetate and inoculated with *Salmonella typhimurium* after 1 day of incubation at 37°C (STD ERR ± 0.015)

EDTA[c] (%)	SL[b] (%)	GDL[a] (%)		
		0	0.25	0.5
0	0	6.03	5.63	5.39
0	1.5	6.03	5.85	5.70
0	3.0	6.05	5.87	5.68
0.05	0	5.99	5.58	5.28
0.05	1.5	6.03	5.85	5.64
0.05	3.0	6.04	5.83	5.64

[a] GDL - glucono-delta-lactone.
[b] SL - sodium lactate.
[c] EDTA - disodium ethylenediaminetetraacetate.

The growth of *S. typhimurium* was significantly affected by GDL*SL (P = 0.0001) and SL*EDTA (P = 0.0024) interactions (Table 5-4). The average *S. typhimurium* counts after 1 day of incubation are presented in Table 5-8. The combination of 0.5% GDL and 3.0% SL exhibited 2.4 $\log_{10}$ CFU/ml of growth. It exhibited 4.3, 4.8 and 3.0 $\log_{10}$ CFU/ml less growth than the 0% GDL-0% SL, 0.5% GDL-0% SL and 0% GDL-3.0% SL treatment combinations, respectively. Similarly, treatment combinations with intermediate levels of GDL and SL were 1 to 3.2 $\log_{10}$ CFU/ml lower in growth than treatments containing only SL or GDL. Combinations of EDTA with 1.5 and 3.0% SL were 0.6 $\log_{10}$ CFU/ml of growth lower than treatments with only SL. *S. typhimurium* counts decreased by 0.4 to 1.5 $\log_{10}$ CFU/ml when EDTA was utilized in combinations of GDL and SL.

Table 5-8. Levels ($\log_{10}$ CFU/ml) of *Salmonella typhimurium* in cooked meat media containing combinations of glucono-delta-lactone, sodium lactate and disodium ethylenediaminetetraacetate after 1 day of incubation at 37°C (STD ERR ± 0.24)

EDTA[c] (%)	SL[b] (%)	GDL[a] (%)		
		0	0.25	0.5
0	0	6.7	7.1	7.2
0	1.5	6.9	5.9	4.6
0	3.0	5.4	3.9	2.4
0.05	0	7.2	7.1	6.9
0.05	1.5	6.3	5.4	3.1
0.05	3.0	4.8	3.1	2.0

[a] GDL - glucono-delta-lactone.
[b] SL - sodium lactate.
[c] EDTA - disodium ethylenediaminetetraacetate.

The results of the statistical analysis on the pH and growth of *E. coli* O157:H7 and *S. typhimurium* in selected treatment combinations during 3 days of incubation are presented in Table 5-9. There were significant (P = 0.0001) treatment by day interactions for both pH and growth in both experiments. For both organisms there was typically a 0.08 to 0.17 increase in the pH during the incubation period (Table 5-10). In the experiment with *S. typhimurium*, there were several treatment combinations that resulted in a 0.04 to 0.08 unit reduction in pH during incubation.

Table 5-9. Probability values (P > F) for the effects of selected combinations of glucono-delta-lactone, sodium lactate and disodium ethylenediaminetetraacetate on the growth and pH of *Escherichia coli* O157:H7 and *Salmonella typhimurium* in cooked meat media for 3 days of incubation at 37°C

Source	*E. coli* O157:H7		*S. typhimurium*	
	pH	CFU	pH	CFU
Replication	0.0001	0.5714	0.0001	0.0001
Treatment	0.0001	0.0001	0.0001	0.0001
Day	0.0001	0.0868	0.0001	0.0785
Treatment*Day	0.0001	0.0001	0.0001	0.0001

The growth of *E. coli* during incubation at 37°C is presented in Table 5-11. The treatment combinations of 0% EDTA-3.0% SL-0.5% GDL, 0.05% EDTA-3.0% SL-0% GDL and 0.05% EDTA-1.5% SL-0.5% GDL exhibited 4.0 to 4.6 $\log_{10}$ CFU/ml of growth at day 3. These treatments were considered bacteriostatic to *E. coli* O157:H7. The treatment combinations of 0.05% EDTA-3.0% SL-0.25% GDL and 0.05% EDTA-3.0% SL-0.5% GDL exhibited 3.8 and 2.6 $\log_{10}$ CFU/ml at day 3, respectively, and were considered bactericidal.

The growth of *S. typhimurium* during incubation at 37°C is also presented in Table 5-11. At day 3, the treatment combinations of 0.05% EDTA-3.0% SL-0% GDL and 0.05% EDTA-1.5% SL-0.25% GDL exhibited 4.5 and 4.9 $\log_{10}$ CFU/ml of growth, respectively and were considered bacteriostatic. Treatment combinations of 0% EDTA-3.0% SL- 0.25% GDL, 0% EDTA-3.0% SL-0.5% GDL, 0.05% EDTA-3.0% GDL-0.25% SL and 0.05% EDTA-3.0% SL-0.5% SL exhibited 1.8 to 3.6 $\log_{10}$ CFU/ml of growth at day 3 and were considered bactericidal.

Table 5-10. The pH values of selected cooked meat media treatments inoculated with *Escherichia coli* O157:H7 and *Salmonella typhimurium* in cooked meat media during a 3 day incubation at 37°C

Treatment combination			Day			
EDTA[c] (%)	SL[b] (%)	GDL[a] (%)	0	1	2	3
E. coli O157:H7 (STD ERR ± 0.009)						
0	3.0	0.25	5.85	5.92	5.94	5.97
0	1.5	0.5	5.65	5.73	5.76	5.78
0	3.0	0.5	5.67	5.76	5.77	5.81
0.05	1.5	0	6.03	6.07	6.05	6.12
0.05	3.0	0	6.02	6.08	6.07	6.10
0.05	1.5	0.25	5.84	5.90	5.91	5.92
0.05	3.0	0.25	5.82	5.91	5.91	5.94
0.05	1.5	0.5	5.64	5.73	5.74	5.76
0.05	3.0	0.5	5.65	5.73	5.74	5.76
S. typhimurium (STD ERR ± 0.013)						
0	3.0	0	6.11	6.05	6.06	6.05
0	1.5	0.25	5.89	5.85	5.83	5.81
0	3.0	0.25	5.86	5.87	5.91	5.93
0	1.5	0.5	5.73	5.70	5.71	5.72
0	3.0	0.5	5.60	5.68	5.74	5.77
0.05	3.0	0	6.07	6.04	6.03	6.03
0.05	1.5	0.25	5.87	5.85	5.84	5.86
0.05	3.0	0.25	5.83	5.83	5.88	5.91
0.05	1.5	0.5	5.65	5.64	5.69	5.71
0.05	3.0	0.5	5.62	5.64	5.71	5.74

[a] GDL - glucono-delta-lactone.

[b] SL - sodium lactate.

[c] EDTA - disodium ethylenediaminetetraacetate.

Table 5-11. The growth ($\log_{10}$ CFU/ml) of *Escherichia coli* O157:H7 and *Salmonella typhimurium* in selected cooked meat media treatments during a 3 day incubation at 37°C

Treatment combination			Day			
EDTA[c] (%)	SL[b] (%)	GDL[a] (%)	0	1	2	3
E. coli O157:H7 (STD ERR ± 0.15)						
0	3.0	0.25	4.5	4.7	5.4	5.6
0	1.5	0.5	4.5	5.8	6.2	6.4
0	3.0	0.5	4.5	4.1	4.4	4.0
0.05	1.5	0	4.5	6.0	6.1	6.2
0.05	3.0	0	4.5	4.3	4.3	4.6
0.05	1.5	0.25	4.5	5.0	5.7	5.9
0.05	3.0	0.25	4.5	4.2	4.2	3.8
0.05	1.5	0.5	4.5	4.8	4.4	4.3
0.05	3.0	0.5	4.5	4.1	3.6	2.6
S. typhimurium (STD ERR ± 0.33)						
0	3.0	0	4.6	5.4	6.2	6.7
0	1.5	0.25	4.6	5.9	6.4	6.7
0	3.0	0.25	4.6	3.9	3.8	3.6
0	1.5	0.5	4.6	4.6	5.3	5.8
0	3.0	0.5	4.6	2.4	1.8	2.0
0.05	3.0	0	4.6	4.8	5.0	4.5
0.05	1.5	0.25	4.6	5.4	4.8	4.9
0.05	3.0	0.25	4.6	3.1	2.7	2.3
0.05	1.5	0.5	4.6	3.1	5.1	5.1
0.05	3.0	0.5	4.6	2.0	1.8	1.8

[a] GDL - glucono-delta-lactone.

[b] SL - sodium lactate.

[c] EDTA - disodium ethylenediaminetetraacetate.

For both *E. coli* O157:H7 and *S. typhimurium* there were significant ($P < 0.05$) GDL*SL and SL*EDTA interactions on organism growth, while there was not a GDL*EDTA interaction effect on growth. The significant interaction of SL with both GDL and EDTA indicated that SL was probably the preservative responsible for inhibiting microbial growth. The increased antimicrobial activity was likely the result of GDL and EDTA effecting characteristics of either the SL or the microorganism being investigated.

Potential modes of synergistic interaction

As discussed in Chapter 2, EDTA's mechanism for enhancing the antimicrobial activity of preservatives is that it binds to divalent cations in the cell membrane of bacteria which increases membrane permeability and increases the penetration of antimicrobial agents into the cell's cytoplasm (Kraniak and Shelef, 1988).

Glucono-delta-lactone is acidic upon hydrolysis, therefore its mechanism of synergistic action is likely reduced pH, which increases the concentration of undissociated lactic acid. According to Giese (1994), the undissociated form of an acid is responsible for its antimicrobial activity. Therefore, as the concentration of undissociated lactic acid increases, the antimicrobial activity of lactic acid also increases. This potential mechanism was investigated by calculating the ratios and percentages of dissociated (A-) and undissociated (HA) lactic acid and the concentration of undissociated lactic acid for each treatment combination and organism. These values are presented in Tables 5-12 and 5-13. Calculations for the [A-]:[HA] ratio were performed using the Henderson-Hasselbach equation ($pH = pK_a + \log [A-]/[HA]$) with the reported pK_a of 3.86 for lactic acid (Segel, 1976). In general, the [A-]:[HA] ratio decreased as GDL increased within each level of SL. This trend was the result of reduced pH associated with increased GDL concentration. The calculations of percent A- and HA were calculated with the assumption that sodium lactate is 79.5% lactic acid (based on molecular weight) and using either the [A-]:[HA] ratio for the particular treatment or [A-]:[HA] ratio for the GDL level at 0% SL. The [A-] ratios for GDL levels at 0% SL were used to calculated the A- and HA concentrations with the intent of compensating for pH changes that occurred as a result of the GDL*SL interaction. For the calculations using the [A-]:[HA] ratio for each treatment combination the percentage of HA doubled as GDL increased from 0 to 0.5% within a given percent lactic acid. For the calculations using the [A-]:[HA] ratios from 0% SL, the percentage of HA in the 0.5% GDL treatments were 3 to 4 times greater than those with 0% GDL, within the same percent lactic acid. The highest calculated percent of undissociated lactic acid was 0.069%.

Table 5-12. Calculated values for dissociated and undissociated lactic acid ratios ([A-]:[HA]), percentages of dissociated and undissociated lactic acid (%A-:%HA) and the concentration of undissociated lactic acid (HA) in treatments containing sodium lactate and glucono-delta-lactone from the *Escherichia coli* O157:H7 experiment

SL[b] (%)	GDL[a] (%)		
	0	0.25	0.5
	[A-]:[HA]	[A-]:[HA]	[A-]:[HA]
0	169.8:1	75.9:1	50.1:1
1.5	169.8:1	114.8:1	74.1:1
3.0	173.8:1	114.8:1	79.4:1
Lactic acid as percent of A- and HA in each treatment combination. Calculation was perform perform performed using [A-]:[HA] for each combination.			
Lactic acid	% A-:% HA	% A-:% HA	% A-:% HA
0	0:0	0:0	0:0
1.2	1.189:0.007 (0.78)[c]	1.190:0.010 (1.11)	1.184:0.016 (1.78)
2.4	2.386:0.014 (1.55)	2.379:0.021 (2.33)	2.370:0.030 (3.33)
Lactic acid as percent of A- and HA in each treatment combination. Calculation was performed using [A-]:[HA] for each GDL level at 0% SL.			
0	0:0	0:0	0:0
1.2	1.189:0.007 (0.78)	1.184:0.016 (1.78)	1.177:0.023 (2.55)
2.4	2.386:0.014 (1.55)	2.369:0.031 (3.44)	2.353:0.047 (5.22)

[a] GDL - glucono-delta-lactone.

[b] SL - sodium lactate.

[c] Concentration of HA (mmol/L).

Table 5-13. Calculated values for dissociated and undissociated lactic acid ratios ([A-]:[HA]), percentages of dissociated and undissociated lactic acid (%A-:%HA) and the concentration of undissociated lactic acid (HA) in treatments containing sodium lactate and glucono-delta-lactone from the *Salmonella typhimurium* experiment

SL[b] (%)	GDL[a] (%)		
	0	0.25	0.5
	[A-]:[HA]	[A-]:[HA]	[A-]:[HA]
0	147.9:1	58.9:1	33.9:1
1.5	147.9:1	97.7:1	69.2:1
3.0	144.5:1	102.3:1	66.1:1
Lactic acid as percent of A- and HA in each treatment combination. Calculation was performed using [A-]:[HA] for each combination.			
Lactic acid	% A-:% HA	% A-:% HA	% A-:% HA
0	0:0	0:0	0:0
1.2	1.192:0.008 (0.89)[c]	1.188:0.012 (1.33)	1.183:0.017 (1.89)
2.4	2.383:0.017 (1.89)	2.377:0.023 (2.55)	2.364:0.036 (4.00)
Lactic acid as percent of A- and HA in each treatment combination. Calculation was performed using [A-]:[HA] for each GDL level at 0% SL.			
0	0:0	0:0	0:0
1.2	1.192:0.008 (0.89)	1.180:0.020 (2.22)	1.166:0.034 (3.77)
2.4	2.384:0.016 (1.78)	2.360:0.040 (4.44)	2.331:0.069 (7.66)

[a] GDL - glucono-delta-lactone.
[b] SL - sodium lactate.
[c] Concentration of HA (mmol/L).

The concentration (mmol/L) of undissociated lactic acid was calculated by converting the percent of undissociated acid to g/L and dividing it with the molecular weight of lactic acid (90.08). The resulting Mol/L was converted to mmol/L. For *E. coli* O157:H7, the concentration of undissociated lactic acid in treatments that contained SL, either alone or in combination with GDL ranged from 0.78 to 3.33 mmol/L when calculated with the [A-]:[HA] ratio for the particular treatment. The undissociated lactic acid concentrations for the same treatments ranged from 0.78 to 5.22 mmol/L when calculated with the [A-]:[HA] ratio for the GDL level at 0% SL. The later method increased the calculated undissociated lactic acid concentration of GDL containing treatments by 0.67 to 1.89 mmol/L, thus compensating for the increase in pH associated with increasing levels of sodium lactate. The GDL-SL combinations that inhibited the growth of *E. coli* O157:H7 contained 1.78 to 5.22 mmol/L of undissociated lactic acid. The combination of 0.5% GDL-3.0% SL was the most inhibitory and contained either 3.33 or 5.22 mmol/L of undissociated lactic acid, depending upon the method of calculating [A-]:[HA].

For *S. typhimurium*, the concentration of undissociated lactic acid in treatments that contained SL, either alone or in combination with GDL ranged from 0.89 to 4.00 mmol/L when calculated with the [A-]:[HA] ratio for the particular treatment. The undissociated lactic acid concentrations for the same treatments ranged from 0.89 to 7.66 mmol/L when calculated with the [A-]:[HA] ratio for the GDL level at 0% SL. The later method increased the calculated undissociated lactic acid concentration of treatments containing GDL by 0.89 to 3.66 mmol/L, thus compensating for the increase in pH associated with increasing levels of sodium lactate. The GDL-SL combinations exhibiting inhibitory or bactericidal activity contained 1.33 to 7.66 mmol/L of undissociated lactic acid. Combinations of 0.25% GDL-3.0% SL and 0.5% GDL-3.0% SL exhibited bactericidal effects and contained 2.55 and 4.00 or 4.44 and 7.66 mmol/L of undissociated lactic acid, respectively, depending upon the method used to calculate [A-]:[HA].

The concentration of undissociated lactic acid required for antimicrobial activity toward *E. coli* O157:H7 and *S. typhimurium* appear to be consistent with what has been reported or extrapolated from data for *L. monocytogenes*, *Y. enterocolitica* and *E. coli* O157:H7. Giannuzzi and Zaritsky (1996) reported that 0.97 to 4.0 mmol/L of undissociated lactic acid inhibited the growth of *L. monocytogenes* in tryptic soy broth at 4 and 10°C. When undissociated lactic acid concentration was increased to 16.0 mmol/L cell death occurred at a rate of 0.3 to 0.4 $\log_{10}$ CFU/ml/day. Brackett (1987) reported that 18 mmol/L of undissociated lactic acid resulted in a 5.5 to 6.5 $\log_{10}$ CFU/ml reduction in *Y. enterocolitica* after incubation in tryptic soy broth for 24 hr at 25°C. Abdul-Raouf et al. (1993) evaluated the survival of *E. coli* O157:H7 in a beef slurry acidified with lactic acid. When the pH was 4.7 and incubation temperature was 5 or 21°C *E. coli* O157:H7 counts decreased. However, when the incubation temperature was increased to 30°C the antimicrobial effects were lost. The undissociated lactic acid concentration at pH 4.7 was calculated to be approximately 2.1 mmol/L.

In addition to the reduced pH associated with GDL, there may be other factors influencing the percent of undissociated lactic acid in each treatment. The pK_a of an acid is affected by the ionic strength of the medium that it is dissolved in. The CMM contained ~0.1 M sodium phosphate (mono- and di-basic) and up to 0.03 M GDL, 0.27 M SL and 0.003 M EDTA, in addition to the proteins and minerals naturally present within the media. All of these components affect the ionic strength of the CMM media and will therefore affect the concentration of undissociated lactic acid.

Conclusions

Combinations of GDL, SL and EDTA exhibited bacteriostatic to bactericidal effects on *E. coli* O157:H7 and *S. typhimurium*. Combinations of EDTA and GDL exhibited a slight but nonpractical inhibitory effect on the growth of *E. coli* O157:H7, but did not exhibit any antimicrobial activity toward *S. typhimurium*. This indicated that SL was the main antimicrobial agent in this experiment and that GDL and EDTA enhanced its antimicrobial activity. The mechanism for the synergistic interaction between EDTA and SL is that EDTA binds to divalent cations in the cell membrane of bacteria which increases membrane permeability and increases the penetration of SL into the cell's cytoplasm (Kraniak and Shelef, 1988). The proposed mechanism for GDL is an equilibrium between GDL and lactic acid (from SL). The pK_as of SL and GDL are 3.86 and 3.7, respectively. GDL has a lower pK_a therefore it will tend to dissociate to the GDL^- and H^+, contributing H^+ to LA^- and forming undissociated lactic acid. Therefore, the concentration of undissociated lactic acid in the media increases. Undissociated lactic acid penetrates cell membranes more readily than dissociated lactic acid. The EDTA*SL and GDL*SL interactions both appear to be the result of EDTA or GDL enhancing the penetration of lactic acid or lactate ions (from SL) into the cell's cytoplasm. Once in the cell, lactic acid/ lactate ions may exhibit antimicrobial activity by acidifying the cytoplasm

(Shelef, 1994), interfering with energy metabolism (Maas et al., 1989) and/or chelating polyvalent cations essential for growth (Weaver and Shelef, 1993; Shelef, 1994).

Comparison of Current Results with Results Obtained in Chapters 3 and 4

There were several differences in the preparation of the CMM between experiments that may be sources of variation in the antimicrobial activity of SL and GDL between the experiments. In Chapters 3 and 4, the CMM pellets were added to the test tubes dry, while in this chapter the CMM was mixed with water then the liquid and pellet portions were separated and dispensed individually. The difference in CMM preparation may have caused slight differences in the concentrations of GDL and SL in the media between experiments. In Chapters 3 and 4, the SL and GDL were incorporated into the CMM prior to sterilizing by autoclaving (15 min at 121°C), while in this chapter these preservatives were filter sterilized and incorporated into the CMM after it was autoclaved. In the current experiment approximately 0.1 M of mono- and di- basic phosphates were incorporated into the CMM to stabilize the solution pH during incubation, while phosphates were not included in the CMM in Chapters 3 and 4.

The growth of *E. coli* O157:H7 and *S. typhimurium* in the presence of GDL and SL were also investigated in Chapters 3 and 4 under conditions similar to this experiment. In Chapter 3, the combination of 0.5% GDL-3.0% SL was the only GDL-SL combination that exhibited inhibitory effects toward *E. coli* O157:H7 ATCC 43895. This combination had counts that were 1.3 to 3.2 $\log_{10}$ CFU/ml lower than the control after 24 hr at 37°C (Figs. 3-1 and 3-3). However, counts were greater than 10^6 CFU/ml after incubation for

48 hr. Similar trends were observed for *S. typhimurium* ATCC 14028 in Chapter 4. The combination of 0.5% GDL and 3.0% SL exhibited 3.5 $\log_{10}$ CFU/ml lower than the control after 24 hr at 37°C (Fig. 4-1). After 48 hr, counts were greater than 10^6 CFU/ml. In both cases, bacteria numbers increased from 2 to 3.5 $\log_{10}$ CFU/ml at inoculation to greater than 6 $\log_{10}$ CFU/ml within 48 hr of incubation.

In the current experiment, combinations of GDL and SL were generally more inhibitory toward *E. coli* O157:H7 ATCC 43895 and *S. typhimurium* ATCC 13311 than in the previous experiments. For *E. coli* O157:H7, the combinations of 0.25% GDL-3.0% SL, 0.5% GDL-1.5% SL and 0.5% GDL-3.0% SL exhibited counts that were 2.2 to 3.9 $\log_{10}$ CFU/ml lower than the control after 24 hr at 37°C (Table 5-6). After incubation for 3 days (Table 5-11), the counts for 0.25% GDL-3.0% SL and 0.5% GDL-1.5% SL increased from 4.5 $\log_{10}$ CFU/ml at inoculation to 5.6 and 6.4 $\log_{10}$ CFU/ml, respectively. During the same incubation, the *E. coli* O157:H7 counts for 0.5% GDL-3.0% SL decreased from 4.5 at inoculation to 4.0 $\log_{10}$ CFU/ml. For *S. typhimurium*, the combinations of 0.5% GDL-1.5% SL, 0% GDL-3.0% SL, 0.25% GDL-3.0% SL and 0.5% GDL-3.0% SL exhibited counts that were 1.3 to 4.3 $\log_{10}$ CFU/ml lower than the control after 24 hr at 37°C (Table 5-8). After incubation for 3 days (Table 5-11), the counts for 0% GDL-3.0% SL and 0.5% GDL-1.5% SL increased from 4.6 $\log_{10}$ CFU/ml at incubation to 6.7 and 5.8 $\log_{10}$ CFU/ml, respectively. During the same incubation, the *S. typhimurium* counts in 0.25% GDL-3.0% SL decreased from 4.6 $\log_{10}$ CFU/ml at inoculation to 3.6 to 2.0 $\log_{10}$ CFU/ml, respectively.

Different methods of preparing the CMM were used between Chapter 5 and Chapters 3 and 4 may have caused slight variations in the concentrations of GDL and SL in each treatment which may have caused slight variations in antimicrobial activity between the experiments. An evaluation of replication as a source of variation in microbial growth within each experiment reveals that the probability values (P > F) were P = 0.0030 and 0.0127 for *E. coli* O157:H7 and *S. typhimurium*, respectively, in Chapter 5 (Table 5-4), P = 0.0001 and 0.0073 for *E. coli* O157:H7 in Chapter 3 (Table 3-1) and P = 0.4576 for *S. typhimurium* in Chapter 4 (Table 4-1). Replication was a source of variation for microbial growth in most of the experiments. These variations could have been the result of slight variations in media preparation or pathogen viability between replications. Although there were variations between replications, the general microbial response to each preservative combination was consistent enough between replications to identify variations caused by each preservative combination. The differences in the antimicrobial activity of GDL and SL between Chapter 5 and Chapters 3 and 4 are extreme in that a given SL-GDL combination may have exhibited mild inhibitory activity in Chapters 3 and 4, but was bactericidal in Chapter 5. This extreme variation is too great to be accounted for by different methods of CMM preparation. In Chapters 3 and 4, the SL and GDL were sterilized by autoclaving, while in this chapter these preservatives were filter sterilized and incorporated into the CMM after it was autoclaved. The exposure to high temperature during autoclaving may have altered the structure of either the SL or GDL. The lactic acid in SL could have been partially self-esterified to form lactic acid lactate because lactic acid lactate is commonly prepared by heating lactic acid at 121°C for 10 hr (Merck and Co., Inc., 1989). It is also possible that lactic acid could have been esterified with other compounds during autoclaving. The development of esters may tend to enhance the antimicrobial activity of lactic acid. For other compounds such as medium chain length fatty acids (12 carbon atoms) esterification to glycerol enhances their inhibitory action toward Gram-positive bacteria and yeasts (Lindsay, 1985). However, sodium lactate was less effective against both *E. coli* O157:H7 and *S. typhimurium* in experiments when it was autoclaved than in experiments when it was not autoclaved.

There is also the potential that autoclaving reduced the antimicrobial activity of SL. However, several researchers have utilized lactic acid and/or lactate salts in various media in which the preservatives were either autoclaved before being added to the media or added to the media before autoclaving. The researchers, media used and organism evaluated were Minor and Marth (1972), tryptic soy broth (TSB), *S. aureus*; Brackett (1987), TSB, *Y. enterocolitica*; Sorrells et al. (1989), TSB, *L. monocytogenes*; Sorrells and Enigl (1990), TSB, *L. monocytogenes*; Shelef and Yang (1991), TSB, comminuted beef or chicken, *L. monocytogenes*; Chen and Shelef (1992), strained beef, *L. monocytogenes*; Weaver and Shelef (1993), pork liver sausage, *L. monocytogenes*; Buchanan et al. (1993), brain heart infusion (BHI) broth, *L. monocytogenes*; Houtsma et al. (1994), peptone-yeast extract medium, *C. botulinum*; Buncic et al. (1995), BHI broth, *L. monocytogenes*; and Kouassi and Shelef (1995), TSB supplemented with 0.6% yeast extract, *L. monocytogenes*. In all of these experiments lactic acid and/or lactate salts were reported to exhibit antimicrobial activity, thus indicating that autoclaving did not destroy their antimicrobial properties. There have also been several researchers who filter sterilized lactic acid prior to its incorporation into various test medium to avoid pH changes which may result from autoclaving. The researchers, media used and organisms evaluated were Ahamad and Marth (1990), tryptose broth, *L. monocytogenes*; Young and Foegeding (1993), BHI broth, *L. monocytogenes*; and Conner and Kotrola (1995), TSB with 0.6% yeast extract, *E. coli* O157:H7. Lactic acid exhibited antimicrobial activity in each of these experiments, indicating that the antimicrobial properties in the previously mentioned studies were not the result of autoclaving.

When subjected to heat, GDL rapidly undergoes hydrolysis to form gluconic acid. Hydrolysis also occurs when GDL is held at room temperature, however the hydrolysis rate is slower at room temperature. If autoclaving enhances the hydrolysis of GDL to gluconic acid, it should result in a more rapid pH reduction and enhanced antimicrobial activity. However, the differences in the antimicrobial activity between this chapter and Chapters 3 and 4 could be interpreted to indicate that autoclaving reduced the antimicrobial activity of GDL. Fernandez et al. (1994) reported that GDL reduced the thermal resistance of *Bacillus thermophillus* spores in acidified mushroom extract cooked to temperatures of 115 to 125°C. Therefore, antimicrobial activity was not lost during heating at high temperatures. Oscroft et al. (1990) filter sterilized GDL in experiments evaluating the inhibition of thermally-stressed *Bacillus* spores by combinations of nisin, pH and organic acids. The treatments were subjected to thermal stress at 95°C for 15 min or 65°C for 60 min. GDL exhibited synergistic antimicrobial activity with nisin, again indicating that the effectiveness of GDL is not lost during exposure to high temperatures. El-Shenaway and Marth (1990) filter sterilized gluconic acid and GDL in experiments evaluating the behavior of *L. monocytogenes* in the presence of gluconic acid or GDL in tryptose broth at 35°C. Concentrations of 0.5, 0.75 and 1.0% gluconic acid exhibited pHs of 4.50, 4.13 and 3.85, respectively. Similarly, the lowest pH of concentrations of 0.5, 0.75 and 1.0% GDL were 4.49, 4.20 and 3.70, respectively. The lowest pH at each GDL concentration was generally obtained within 4 to 6 hrs at 35°C and was similar in pH to the corresponding concentration of gluconic acid. The similar pH between GDL and gluconic acid indicates that GDL completely hydrolyses to form gluconic acid within 6 hr. The behavior of *L. monocytogenes* was similar in GDL and gluconic acid with rapid growth occurring after a 24 hr lag phase in 0.5% and reductions in cell numbers occurring in 0.75 and 1.0%. Overall, these experiments indicate that GDL is an effective antimicrobial agent and that its effectiveness is not likely to be altered by autoclaving.

Another potential source of the increased antimicrobial activity observed for SL and GDL in this experiment is the addition of phosphate. Wagner (1986) reviewed the antimicrobial activity of polyphosphates and their use as antibotulinal agents in meats. Similarly, Shelef and Seiter (1993) included polyphosphates in a review of indirect antimicrobials. Dickson et al. (1994) reported that a trisodium phosphate dip (8-12% at 25-55°C) reduced the populations of *S. typhimurium* and *E. coli* O157:H7 on artificially contaminated beef by 1 to 1.5 $\log_{10}$ cycles and 2 to 2.5 $\log_{10}$ cycles on lean and adipose tissue, respectively. The antimicrobial activity is generally believed to be the result of polyphosphates chelating cations essential for microbial growth (Shelef and Seiter, 1993). Long chain polyphosphates are the best sequestering agents for light metal ions such as calcium and magnesium, however all phosphates sequester metal ions (Wagner, 1986).

In this experiment, the sequestering of essential cations by phosphate may have enhanced the antimicrobial activity of SL and GDL.

In summary, the difference in the antimicrobial activity of GDL and SL between Chapter 5 and Chapters 3 and 4 was likely the result of incorporating mono- and di-phosphates into the CMM in Chapter 5. Differences in CMM preparation between Chapter 5 and Chapters 3 and 4 may have caused slight variations in the results of each experiment. However, replication was a source of variation for most of the experiments. The variations within experiments did not effect the general microbial growth trends within each experiment. The differences between experiments were that a given SL-GDL combination may have exhibited mild inhibitory activity in Chapters 3 and 4, but was bactericidal in Chapter 5. Such variations were too extreme to be accounted for by different methods of CMM preparation. Autoclaving SL and GDL, as was done in Chapters 3 and 4, would tend to increase the antimicrobial activity of both preservatives if it affected them at all. However, SL and GDL exhibited stronger antimicrobial activity in Chapter 5 than Chapters 3 and 4, indicating that autoclaving did not cause the difference between experiments. Phosphates have been shown to exhibit antimicrobial activity toward a variety of pathogens (Wagner, 1986; Dickson et al., 1994) and have been identified as indirect antimicrobials (Shelef and Seiter, 1993). Therefore, the addition of mono- and di- basic phosphates to the CMM in Chapter 5 is the most likely cause of the difference in the antimicrobial activity of SL and GDL combinations between Chapter 5 and Chapters 3 and 4.

CHAPTER 6

THE UTILIZATION OF SODIUM LACTATE AND GLUCONO-DELTA-LACTONE IN NONREFRIGERATED AND REFRIGERATED, RESTRUCTURED PORK ROLLS

Introduction

The effects of Nisaplin (NN), sodium lactate (SL), glucono-delta-lactone (GDL), ethylenediaminetetraacetic acid (EDTA) and their combinations on the growth of various foodborne pathogens were discussed in Chapters 3, 4 and 5. Combinations of SL and GDL inhibited the growth of *Escherichia coli* O157:H7, *Salmonella typhimurium*, *Yersinia enterocolitica*, *Listeria monocytogenes* and *Staphylococcus aureus*. Singularly, NN was bactericidal to *L. monocytogenes* and *S. aureus*. It also exhibited a slight reduction in the growth of *E. coli* O157:H7. However, it did not affect the growth of *S. typhimurium* or *Y. enterocolitica*. Combinations of NN with GDL resulted in an antagonistic interaction which enhanced the growth of *S. aureus*. Similarly, combinations of NN with GDL and SL enhanced the growth of *E. coli* O157:H7.

Nisaplin's narrow range of antimicrobial activity and antagonistic interaction with SL and GDL warranted its removal from the list of preservatives and preservative combinations that may be useful to develop a shelf-stable noncured meat product.

In Chapters 3 and 5, 0.05, 0.372 and 0.745% EDTA exhibited synergistic interactions with combinations of GDL and SL to inhibit the growth of *E. coli* O157:H7 and *S. typhimurium*. However, when the EDTA concentration was reduced to 0.0036 and

0.0072% the interactions with GDL and SL were not apparent. The U.S. Food and Drug Administration (FDA) limits the use of EDTA in meat products to 0.0072% (USFDA, HHS, 1995). EDTA will not exhibit antimicrobial activity when used according to the current FDA restrictions, therefore EDTA was not evaluated for its preservative characteristics in a nonrefrigerated, noncured restructured pork product.

Sodium lactate has been shown to delay the growth of *L. monocytogenes*, *Pseudomonas fragi*, *Clostridium perfringens*, *S. typhimurium* and *E. coli* O157:H7 and the toxin production of *Clostridium botulinum* (Maas et al., 1989; Harmayani et al., 1991; Shelef and Yang, 1991; Weaver and Shelef, 1993; Houtsma et al., 1994; Miller and Acuff, 1994; Wederquist, 1994). The USFDA recognizes SL as a GRAS ingredient for direct use in human foods (USFDA, HHS, 1995). Papadopoulos et al. (1991) reported that consumers preferred the overall flavor and beef flavor of beef roasts containing 3.0% SL when compared to roasts with no SL.

Glucono-delta-lactone (gluconic acid lactone) is commonly used in some fermented sausages at 0.5% and is allowed in Genoa salami at 1.0% (Romans et al., 1977). It is an acidulant and does not impart strong acid flavors to food. El-Shenawy and Marth (1990) observed that 0.4 and 1.5% GDL reduced the growth of *L. monocytogenes* in tryptose broth and reconstituted milk, respectively, at 13°C. At 0.4 to 1.0%, GDL reduced the growth of *S. aureus* in Italian dry sausage (Metaxopolous et al., 1981). Similarly, 0.5% GDL inhibited the growth of lactic acid bacteria in pork liver pate' (Madden, 1989).

Qvist et al. (1994) produced bologna style sausage with four formulations: a) standard commercial formulation (control), b) 2% SL with no GDL, c) 2% SL and 0.25%

GDL and d) 2% SL and 0.5% GDL. Sliced sausage was inoculated with a mixture of *L. monocytogenes* serotype 1 and 4 at approximately 100 CFU/g. The product was vacuum packaged and stored at 5 or 10°C for 35 days. Rapid growth occurred in the control stored at 5 and 10°C and the 2% SL treatment stored at 10°C. The 2% SL treatment stored at 5°C suppressed growth for 28 days. Addition of GDL at 0.25 and 0.5% inhibited growth for 35 days at both storage temperatures.

The previously mentioned research (Chapters 3, 4 and 5) has shown combinations of SL and GDL to effectively suppress the growth of *E. coli* O157:H7, *S. typhimurium*, *Y. enterocolitica*, *L. monocytogenes* and *S. aureus* incubated in cooked meat media at 37°C. Thus, a study was performed to evaluate the effectiveness of SL and GDL for preserving restructured pork rolls under refrigerated and nonrefrigerated storage conditions. The storage characteristics of pork rolls preserved with SL and GDL were also compared with pork rolls preserved with sodium nitrite.

Materials and Methods

Experimental design

The same combinations of GDL and SL utilized in Chapters 3, 4 and 5 were applied to restructured pork rolls. These were compared to two controls, one with sodium nitrite and the other with only salt and sodium tripolyphosphate. All treatments contained salt and sodium tripolyphosphate. The treatments included 1) control which contained only salt and sodium tripolyphosphate, 2) nitrite control which contained sodium nitrite (0.0156%) and sodium erythorbate (0.055%), 3) 0.25% GDL-1.5% SL, 4) 0.25% GDL-

3.0% SL, 5) 0.5% GDL-1.5% SL and 6) 0.5% GDL-3.0% SL. The pork rolls were produced on three different days to form three replications. After packaging, the pork rolls were divided into two groups. One group was stored under nonrefrigerated (18 to 22°C) conditions and the other group was stored under refrigerated (0 to 3°C) conditions.

Microbial, 2-thiobarbituric acid (TBA) and pH analysis were performed on nonrefrigerated samples at 0, 3, 6, 9, 12 and 15 days of storage. Water activity was evaluated at 0, 6 and 12 days of storage on the nonrefrigerated samples. Microbial, TBA, water activity and pH analysis were performed on refrigerated samples at 0, 4 and 8 weeks of storage. Product composition was determined on samples from day/week 0 of each replication. Three pork rolls were analyzed from each treatment at each evaluation time.

Pork roll processing

Restructured pork rolls were produced from conventionally processed hams, loins and shoulders from sow carcasses trimmed of excess fat and connective tissue. The pork rolls were formulated to consist of 0.75% sodium chloride, 0.4% sodium tripolyphosphate and 5% final fat content. Water was added at 11% of the raw product's weight to compensate for an anticipated 90% cooking yield. The meat block contained 80% coarse ground lean and 20% emulsion. The emulsion was prepared with lean shanks and knuckles, half of the salt and half of the water from each treatment. The emulsion, coarse ground lean and treatment specific ingredients were mixed for 20 minutes under vacuum (-0.8 bar) in a 30 kg capacity vacuum mixer (Keebler, Chicago, IL). The mixture was then stuffed using a Vemag Robot 500, type 128, vacuum stuffer (Robert Reiser and Co., Inc., Canton, MO 02021) into 9.5 cm diameter regular fibrous casings (6 1/2KN X clear semi finished, Viskase, Chicago, IL). The pork rolls were held at 0 to 3°C overnight to allow for protein binding and further ingredient diffusion. Rolls were cooked in a Vortron smokehouse (Model #500, Vortron Inc., Beloit, WI) to an average internal temperature of 71°C. Internal temperature of the first replication was monitored with a meat thermometer. In the second and third replications, smokehouse air temperature and pork roll internal temperatures were monitored with a Multipoint Recorder/Logger (Model MRL-25032-BD-RC-64, Esterline Angus Co., Indianapolis, IN) with copper-constantan thermocouples. Final smokehouse air temperature had a 5°C range and final product internal temperatures had a 5 to 10°C range. The pork rolls were chilled overnight (0 to 3°C), vacuum packaged in Cryovac #B450 bags (Cryovac, W.R. Grace and Co., Duncan, SC) and heat shrunk. The rolls were divided into two groups for storage: nonrefrigerated (18 to 22°C) and refrigerated (0 to 3°C).

Cooking yields

Cooking yields for each treatment were calculated by dividing the cooked product weight of an entire treatment by its raw stuffed weight and multiplying by 100.

Microbial analysis

Microbial analysis included enumerating aerobic and anaerobic organisms and evaluating for the presence of *Salmonella* spp. Enumeration involved aseptically transferring 25g of pork roll into a sterile stomacher bag, making a 1:10 dilution with 0.1% peptone water, pummelled homogenizing with a stomacher (Model #400, Tekmar Co., Cincinnati, OH) for 1 min and 15 sec, and making serial dilutions with 0.1% peptone water. Dilutions were then transferred and spread onto selective media. Aerobic organisms were enumerated on plate count agar (Difco #0479-17-3). Inoculated plate count agar medium was incubated at 35°C for 48 hr to enumerate mesophilic organisms or at 7°C for 10 days to enumerate psychrotrophic organisms. Psychrotrophic counts were not determined on the nonrefrigerated products. Anaerobic organisms were enumerated on anaerobic agar (Difco #0536-17-4) incubated in Gas Pak chambers with an atmosphere containing H_2 and CO_2 at 35°C for 3 days.

The presence of *Salmonella* species was determined according to the FDA approved method of the *Bacteriological Analytical Manual* (Andrews et al., 1992; Flowers et al., 1992) for the isolation and identification of foodborne *Salmonella* (Appendix B). A 25 g sample was pre-enriched in a 1:10 dilution of lactose broth (Difco #0004-17-0), pH 6.0 to 7.0, incubated for 24 hr at 35°C. Replicate 1 ml portions of lactose broth were selectively enriched in 10 ml of selenite cystine (Difco #0687-15-3) and tetrathionate (Difco #0104-17-6) broths (24 hr at 35°C). Selenite cystine and tetrathionate tubes were vortexed and streaked onto bismuth sulfite and xyloselysine desoxylate (XLD, Difco #0788-01-7) plates and incubated for 24 and 48 hr at 35°C. *Salmonella* colonies appeared black with/without metallic sheen on bismuth sulfite and pink or yellow with/without black centers on XLD. Suspect colonies were confirmed on triple sugar iron (Difco #0265-01-9) and lysine iron (Difco #0849-01-4) agar slants (24 hr at 35°C). Positive *Salmonella* exhibited alkaline (red) slants and acid (yellow) butts on triple sugar iron agar and alkaline (purple) slants and butts on lysine iron agar.

Chemical analysis

Moisture, fat, protein and ash were determined using three pork rolls from each treatment. Analysis procedures for moisture (24.003), fat (18.044), protein (24.037) and ash (18.025) were conducted as outlined in the Official Methods of Analysis of the AOAC (AOAC, 1990).

Water activity (a_w) was measured with an Aqualab model CX2 (Decagon Devices, Inc., Pullman, WA) water activity measuring device. Two samples (0.5 cm thick X 2.5 cm long X 1.3 cm wide) were obtained from the interior of each pork roll. These were placed in covered sample cups and held at room temperature (20 to 23°C) for 1 hour. Sample cups were opened and placed in the Aqualab to analyze water activity and temperature.

Pork roll pH was evaluated with a portable Oyster (Extech Instruments Corporation, Waltham, MA) pH meter. The probe was inserted into the cut surface of the pork rolls. The pH was evaluated at three different locations which were averaged to obtain one pH value for each pork roll.

A 1.3 cm slice was removed from the center of each pork roll and chopped in a food processor for 1.5 to 2 minutes. The chopped meat was evaluated for oxidative rancidity using 2-thiobarbituric acid (TBA) as described by Rhee (1978). Duplicate ten gram samples of chopped noncured pork roll were blended in 47.5 ml cold deionized water and 5 ml antioxidant suspension (0.5% [W/V] propyl gallate and 0.5% [W/V] EDTA in distilled water) for three 10 s bursts with a hand held Biomixer (M122, Biospec Products, Bartlesville, OK). Pork rolls containing nitrite were blended in 47 ml cold deionized water, 5 ml antioxidant and 1 ml sulfanilamide reagent (0.5% [W/V] sulfanilamide in 20% [V/V] HCl). The homogenate was quantitatively transferred by rinsing with an additional 45 ml cold deionized water into a Kjeldhal flask containing 2.5 ml of 4 M HCl to adjust final pH to 1.5. Samples containing nitrite were transferred into flasks containing 2.0 ml of 4 M HCl to adjust the final pH. The homogenate was distilled to yield 50 ml of distillate. Duplicate 5 ml aliquots of distillate were mixed with 5 ml of TBA reagent (0.02 M 2-thiobarbituric acid in 90% [V/V] glacial acetic acid) and heated in boiling water for 35 min. After heating, the samples were cooled in tap water for 10 min and their absorbance was read at 535 nm using a Lambda 3 UV/VIS spectrophotometer (Perkin-Elmer Corporation, Oak Brook, IL). The absorbance was multiplied by 7.8 to convert to mg of malonaldehyde per 1000 g of meat.

Sensory analysis

An 18 member sensory panel received training to evaluate juiciness, pork flavor intensity, saltiness and off-flavors. Panelists were trained for juiciness and pork flavor intensity evaluations with *semimembranosus* and *semitendinosus* muscles from market weight and mature pigs, cooked to different levels of doneness. Panelists were trained for saltiness and off-flavors by tasting solutions of salt (0, 1 and 2% W/V), SL (3% V/V) and GDL (0.5% W/V) and by tasting noncured pork products with different levels of these ingredients.

The trained sensory panel evaluated the products at day 0 of each replication. Samples were served at room temperature without reheating. At each session, panelists were served 4 samples, water for rinsing their pallets and a 1% salt solution as a saltiness standard score of 4 (salty). The numerical scales for sensory evaluation were similar to those presented in the *Research Guidelines for Cookery, Sensory Evaluation and Instrumental Tenderness Measurements of Fresh Meat* (AMSA, 1995) and used by Ziprin et al. (1994) and Miller et al. (1995). Pork rolls were evaluated with the following trait and rating scales: juiciness (8 = extremely juicy, 7 = very juicy, 6 = moderately juicy, 5 = slightly juicy, 4 = slightly dry, 3 = moderately dry, 2 = very dry and 1 = extremely dry); pork flavor intensity (8 = extremely intense, 7 = very intense, 6 = moderately intense, 5 = slightly intense, 4 = slightly bland, 3 = moderately bland, 2 = very bland and 1 = extremely bland); saltiness (8 = none detected, 7 = threshold; barely detected, 6 = traces, 5 = slightly salty, 4 = salty, 3 = moderately salty, 2 = very salty and 1 = extremely salty); and off-flavors (6 = none detected, 5 = threshold; barely detected, 4 = slight off-flavor, 3 = moderate off-flavor, 2 = strong off-flavor and 1 = extreme off-flavor).

Statistical analysis

Statistical analysis was performed with the general linear models program (PROC GLM) of SAS (SAS Institute, 1985). Treatment effects were identified with analysis of variance. Treatment differences were determined using the least squared means pdiff statement. Sensory panel results were analyzed by analysis of variance. When significant ($P < 0.05$) treatment effects were observed, the differences among means were identified with the Student-Newman-Keuls test.

Results and Discussion

Cooking yields

The average cooking yield was 81.9% which was 8% lower than anticipated. Both SL and GDL are water soluble, therefore a small portion of each compound may have been lost during cooking. In such a case, the final product compositions would be similar to the formulation. However, if small quantities of GDL and SL were not lost during cooking, the final products may have contained as high as 1.65 and 3.29% SL for the 1.5 and 3.0% SL formulations, respectively, and 0.27 and 0.55% GDL for the 0.25 and 0.5% GDL formulations, respectively. Such an increase in the SL or GDL content of the products may have increased the antimicrobial activity of each treatment combination.

There were no significant ($P > 0.05$) differences in the cooking yields between treatments as shown in Table 6-1; however, there were differences ($P < 0.001$) in the cooking yields between replications (Table 6-2). The cooking yield for the first replication was 4.5% higher than the yields for the second and third replications. The pork rolls' internal temperature was not as thoroughly monitored during the first replication as it was during the second and third replications. This may have resulted in the first replication being heated to a lower internal temperature than those that followed. Cooking yields are dependent upon endpoint temperature, with higher endpoint temperatures resulting in lower cooking yields.

Table 6-1. Chemical composition and cooking yields of restructured pork rolls by treatment

Treatment	Moisture (%)	Fat (%)	Protein (%)	Ash (%)	Cooking Yield (%)
Control	70.3[b]	4.8[b]	21.9[b]	2.2[d]	81.7
NO_2 Control	70.5[b]	4.7[b,c]	21.6[b]	2.3[d]	82.1
0.25% GDL-1.5% SL[a]	69.8[c]	4.3[c,d]	20.9[c,d]	2.9[b]	82.4
0.5% GDL-1.5% SL	70.3[b]	4.2[d]	21.5[b,c]	2.7[c]	81.4
0.25% GDL-3.0% SL	69.3[c,d]	4.4[b,c,d]	20.9[c,d]	2.9[b]	82.3
0.5% GDL-3.0% SL	68.9[d]	4.8[b]	20.7[d]	3.1[b]	81.9

[a] GDL - glucono-delta-lactone, SL - sodium lactate.

[b,c,d,e] Values in same column with different superscripts are significantly different ($P < 0.001$).

Table 6-2. Chemical composition and cooking yields of restructured pork rolls by replication

Replication	Moisture (%)	Fat (%)	Protein (%)	Ash (%)	Cooking Yield (%)
1	70.2[a]	6.2[a]	19.4[c]	2.4[b]	85.3[a]
2	70.1[a]	3.3[c]	22.4[a]	2.8[a]	80.8[b]
3	69.3[b]	4.0[b]	21.9[b]	2.8[a]	79.7[c]
Average	69.86	4.52	21.26	2.66	81.9

[a,b,c] Values in same column with different superscripts are significantly different ($P < 0.001$).

Pork roll composition

Treatment averages for pork roll composition are presented in Table 6-1. They ranged from 68.9 to 70.5% for moisture content, 4.2 to 4.8% for fat content, 20.7 to

21.9% for protein content and 2.2 to 3.3% for ash content. Moisture, fat and protein contents exhibited significant ($P < 0.001$) but small differences. Variations in moisture and protein appear to be related to the amount of additives in the product formulation. Pork rolls with GDL and SL generally contained less moisture and protein than the controls. Significant differences ($P < 0.001$) were also observed for ash content. As SL content was increased from 0 to 3.0% of the product composition, ash content increased from 2.18 to 3.11%.

Replication averages for pork roll composition (Table 6-2) ranged from 69.3 to 70.2% for moisture content, 3.3 to 6.2% for fat content, 19.4 to 22.4% for protein content and 2.4 to 2.8% for ash content. The first replication had the highest average fat and moisture contents and the lowest average protein content. The higher average fat content was likely the result of slight variations in the composition of the raw materials on each processing day. Replication 1 had a moisture:protein (M:P) ratio of 3.62 while replications 2 and 3 had M:P ratios of approximately 3.15. The higher M:P ratio and previously mentioned higher cooking yield for the first replication indicates that it was probably cooked to a slightly lower internal temperature than the second and third replications. Overall the average composition was 69.9% moisture, 4.5% fat, 21.3% protein and 2.7% ash.

Sensory evaluation

Sensory evaluation was only performed at day 0 of each replication, therefore no storage effects were reported. The mean scores of the pork rolls are presented in Table 6-3. The sensory panel rated all of the pork roll treatments as moderately juicy, slightly intense in pork flavor, slightly salty to salty and threshold to slight in off-flavors. The nitrite control was less ($P < 0.05$) intense in pork flavor than the GDL and SL treatments. The GDL and SL treatments were scored as more ($P < 0.05$) salty than the controls. Other researchers (Brewer et al., 1991; Papodopolous et al., 1991b; Brewer et al., 1992) have also observed that SL intensifies saltiness in fresh pork sausage, roast beef and beef bologna. The 3.0% SL treatments exhibited more ($P < 0.05$) off-flavors than the controls. The observation of off-flavors tended to increase as GDL and SL levels were increased. The most commonly reported off-flavors were acid and bitter.

Table 6-3. Sensory scores for restructured pork rolls

Treatment	Juiciness[a]	Pork Flavor Intensity[a]	Saltiness[b]	Off-flavors[c]
Control	6.0	$5.6^{e,f}$	5.4^{e}	5.5^{e}
NO_2 Control	6.3	5.3^{f}	5.3^{e}	5.7^{e}
0.25% GDL-1.5% SL[d]	6.2	5.9^{e}	4.9^{f}	$5.2^{e,f,g}$
0.5% GDL-1.5% SL	6.2	5.9^{e}	4.4^{f}	$5.3^{e,f}$
0.25% GDL-3.0% SL	6.0	5.8^{e}	4.5^{f}	4.8^{g}
0.5% GDL-3.0% SL	6.0	5.8^{e}	4.6^{f}	$4.9^{f,g}$

[a] Juiciness, pork flavor intensity: 8=extremely juicy or intense, 7=very juicy or intense, 6=moderately juicy or intense, 5=slightly juicy or intense, 4=slightly dry or bland, 3=moderately dry or bland, 2=very dry or bland and 1=extremely dry or bland.

[b] Saltiness: 8=none detected, 7=threshold; barely detected, 6=traces, 5=slightly salty, 4 = salty, 3=moderately salty, 2=very salty and 1=extremely salty.

[c] Off-flavors: 6=none detected, 5=threshold; barely detected, 4=slight off-flavor, 3=moderate off-flavor, 2=strong off-flavor and 1=extreme off-flavor.

[d] GDL - glucono-delta-lactone, SL - sodium lactate.

[e,f,g] Values in the same column with different superscripts are significantly different ($P < 0.05$).

Nonrefrigerated storage

Microbial analysis

Statistical analysis of aerobic and anaerobic growth during nonrefrigerated storage (Table 6-4) indicated that growth was affected ($P < 0.0001$) by replication, treatment, day and treatment by day interactions. Aerobic and anaerobic growth followed similar trends (Figs. 6-1, 6-2). The control and the nitrite control exhibited rapid growth of both aerobic and anaerobic organisms during the first nine days of incubation. They exhibited greater than 10^5 CFU/g by the twelfth day of storage. Many of the control and nitrite control packages were bloated by the twelfth day of storage, indicating gas production by the organisms present. When these packages were opened for microbial analysis strong spoiled meat odors were present. All of the SL and GDL combinations maintained less than 10^2 CFU/g for 12 days of storage. SL and GDL combinations were effective at preventing microbial growth for 15 days of nonrefrigerated storage.

Table 6-4. Probability values ($P > F$) for sources of variation in microbial growth during nonrefrigerated (18 to 22°C) storage

Source	Aerobic growth	Anaerobic growth	Presence of *Salmonella*
Replication	0.0001	0.0001	0.2875
Treatment	0.0001	0.0001	0.0001
Day	0.0001	0.0001	0.0001
Treatment*day	0.0001	0.0001	0.0979

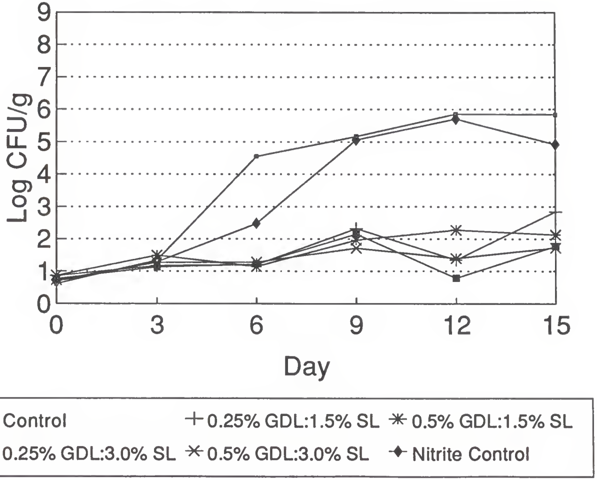

Fig. 6-1—The growth of aerobic mesophiles on restructured pork rolls during non-refrigerated (18 to 22°C) storage (GDL - glucono-delta-lactone; SL - sodium lactate) (STD ERR ± 0.40, n = 9).

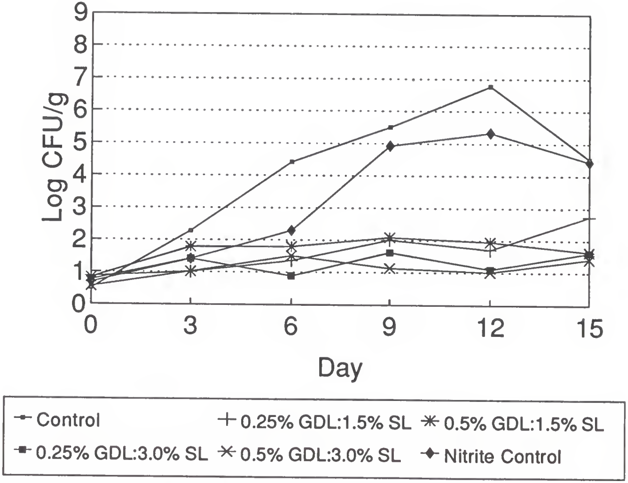

Fig. 6-2—The growth of anaerobic mesophiles on restructured pork rolls during non-refrigerated (18 to 22°C) storage (GDL - glucono-delta-lactone; SL - sodium lactate) (STD ERR ± 0.40, n = 9).

Statistical analysis identified treatment and day as sources of variation ($P < 0.0001$) for the presence of *Salmonella* (Table 6-4). *Salmonella* was observed in all treatments. The highest frequency was in the control and nitrite control at 44 and 40%, respectively (Table 6-5). The frequency in SL and GDL treated pork rolls was 11 to 21%. *Salmonella* was not identified in any treatment at day 0 (Table 6-6). During nonrefrigerated storage, *Salmonella* appeared sporadically. Its frequency at days 3 through 15 was 20 to 35%. The presence of *Salmonella* in the nonrefrigerated pork rolls may be the result of slightly insufficient heating combined with elevated storage temperature, which allowed injured organisms to survive and proliferate during storage. The presence of *Salmonella* in all treatments and its limited growth in SL and GDL treatments suggests that combinations of GDL and SL suppress the resuscitation of heat injured *Salmonella* in cooked nonrefrigerated pork rolls. The survival of *Salmonella* in noncured, nonrefrigerated pork rolls could possibly be reduced by increasing the end-point temperature by 3-5°C or increasing the holding time to several minutes at 71°C.

Table 6-5. The frequency of *Salmonella* in nonrefrigerated, restructured pork rolls by treatment

Treatment	No. of Samples	*Salmonella* Positive Samples	
		No.	(%)
Control	45	20^c	(44)
NO_2 Control	52	21^c	(40)
0.25% GDL-1.5% SL[a]	53	$11^{c,d}$	(21)
0.5% GDL-1.5% SL	54	6^d	(11)
0.25% GDL-3.0% SL	54	9^d	(19)
0.5% GDL-3.0% SL	54	7^d	(13)

[a] GDL- glucono-delta-lactone, SL - sodium lactate.

[c,d] Values in same column with different superscripts are significantly different ($P < 0.001$).

Table 6-6. The frequency of *Salmonella* in nonrefrigerated, restructured pork rolls on a daily basis

Day	No. of Samples	*Salmonella* Positive Samples	
		No.	(%)
0	54	0^b	(0)
3	54	16^a	(30)
6	54	15^a	(28)
9	54	16^a	(30)
12	51	18^a	(35)
15	45	9^a	(21)

[a,b,c] Values in same column with different superscripts are significantly different ($P < 0.001$).

Chemical analysis

TBA analysis of the nonrefrigerated pork rolls exhibited a significant ($P < 0.0001$) treatment by day interaction as shown in Table 6-7. The TBA values as a function of treatment and day are presented in Fig. 6-3. The interaction appeared to be the result of the control, 0.25% GDL-1.5% SL and 0.5% GDL-1.5% SL treatments exhibiting higher ($P < 0.0005$) TBA values than the other treatments at day 3. Similarly, at day 15 the control exhibited higher ($P < 0.05$) TBA values than all other treatments with the exception of the 0.5% GDL-1.5% SL combination. The TBA values for the nitrite control were consistently less than or equal to the TBA values for the other treatments, indicating that sodium nitrite was more effective at inhibiting lipid oxidation than combinations of GDL and SL. TBA values were less than 0.30 mg/1000 g of meat during the entire storage period which indicated that rancidity was not a problem in the pork rolls. The low levels of lipid oxidation are likely the result of the product's low fat content.

Table 6-7. Probability values ($P > F$) for sources of variation in a_w, pH and 2-thiobarbituric acid (TBA) values during nonrefrigerated (18 to 22°C) storage

Source	a_w	pH	TBA
Replication	0.0032	0.0001	0.0001
Treatment	0.0001	0.0001	0.0001
Day	0.0001	0.0001	0.0001
Treatment*day	0.5268	0.0001	0.0001

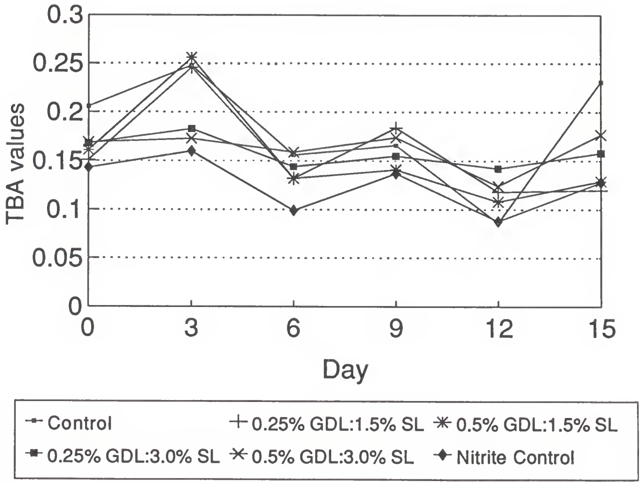

Fig. 6-3—Mean 2-thiobarbituric acid (TBA) values (mg malonaldehyde/kg meat) of restructured pork rolls during non-refrigerated (18 to 22°C) storage (GDL - glucono-delta-lactone; SL - sodium lactate) (STD ERR ± 0.015, n = 9).

Water activity was affected ($P < 0.0001$) by treatment and day of storage (Table 6-7). The mean a_w for nonrefrigerated, restructured pork rolls by treatment and day are presented in Table 6-8. On a treatment basis, the water activity values for the controls were higher ($P < 0.05$) than the SL and GDL treatments. SL has been identified as a humectant and was the likely cause of the reduction in a_w. On a day basis, water activity significantly ($P < 0.0001$) declined from day 0 to day 12, however, the a_w values were not practically different. All the water activity values were within the growth range of both spoilage and pathogenic bacteria and the differences were small. However, the slight reduction in a_w caused by GDL and SL may have contributed to the preservative effects that were observed during nonrefrigerated storage.

Table 6-8. The mean a_w of nonrefrigerated, restructured pork rolls by treatment and day

Treatment	a_w	Day	a_w
Control	0.979^b	0	0.975^b
NO_2 Control	0.979^b	6	0.973^c
0.25% GDL-1.5% SL[a]	0.972^c	12	0.972^d
0.5% GDL-1.5% SL	0.974^c		
0.25% GDL-3.0%SL	0.970^c		
0.5% GDL-3.0%SL	0.968^b		

[a] GDL - glucono-delta-lactone, SL - sodium lactate.

[b,c,d] Values in same column with different superscripts are significantly different ($P < 0.001$).

Statistical analysis of pH revealed a significant ($P < 0.0001$) treatment by day interaction as shown in Table 6-7. The average pH of the nonrefrigerated, restructured pork rolls as a function of treatment and day of storage are presented in Fig. 6-4. The treatment by day interaction appeared to be the result of the 0.5% GDL-3.0% SL treatment exhibiting a 0.1 pH unit increase (pH 5.9 to 6.0) from day 9 to day 15, while the 0.5% GDL-1.5% SL treatment maintained a pH of 5.9. With the exception of the 0.5% GDL-3.0% SL treatment at days 9 through 15, the treatments containing 0.5% GDL were 0.1 pH units lower than the controls and 0.25% GDL treatments. The differences in pH caused by the addition of 0.5% GDL were small, however reduced pH may have contributed to enhanced preservation.

The undissociated lactic acid concentrations for the GDL and SL treated pork rolls ranged from 0.87 to 2.42 mmol/kg of meat or 1.25 to 3.51 mmol/L of moisture in the meat (Table 6-9). The inhibition of pathogenic bacteria has been reported to begin at similar levels of undissociated lactic acid. The growth of *L. monocytogenes* was inhibited by 0.97 mmol/L (Giannuzzi and Zaritzky, 1996). Brackett (1987) reported bactericidal effects toward *Y. enterocolitica* to begin occurring between 2 and 18 mmol/L. At 21°C, 2.1 mmol/L reduced the numbers of *E. coli* O157:H7. However, at 30°C, *E. coli* O157:H7 numbers increased in 2.1 mmol/L of undissociated lactic acid. In chapter 5, inhibitory effects were observed on the growth *E. coli* O157:H7 and *S. typhimurium* when the undissociated lactic acid concentration was 1.78 and 1.33 mmol/L or greater, respectively. The concentrations of undissociated lactic acid in the pork rolls appears to be near the minimum levels required to inhibit the growth of pathogenic bacteria.

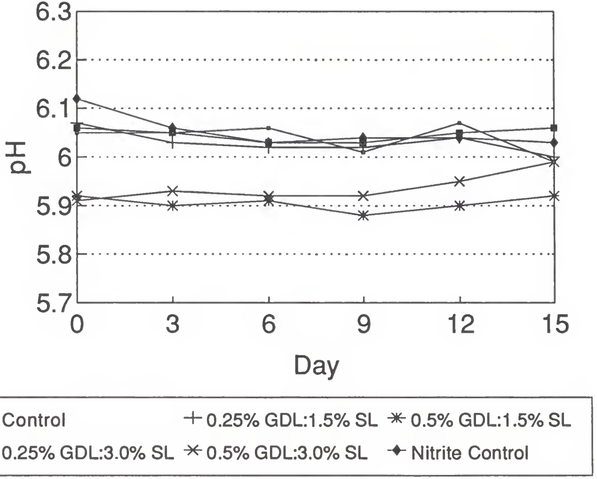

Fig. 6-4—Mean pH of restructured pork rolls during non-refrigerated (18 to 22°C) storage (GDL - glucono-delta-lactone; SL - sodium lactate) (STD ERR ± 0.014, n = 9).

Table 6-9. The average undissociated lactic acid concentration of restructured pork rolls containing sodium lactate and glucono-delta-lactone during 15 days of nonrefrigerated storage

Treatment	pH	Total Lactic Acid Concentration (%)	Undissociated Acid Fraction	Undissociated Acid Concentration	
				mmol/Kg meat	mmol/L of moisture
0.25% GDL-1.5% SL[a]	6.05	1.2	0.0065	0.87	1.25
0.5% GDL-1.5% SL	5.93	1.2	0.0085	1.13	1.61
0.25% GDL-3.0%SL	6.03	2.4	0.0068	1.81	2.61
0.5% GDL-3.0%SL	5.90	2.4	0.0091	2.42	3.51

[a]GDL - glucono-delta-lactone, SL - sodium lactate.

At 0.5%, GDL only reduced pH by 0.10 when compared to controls and treatments with 0.25% GDL. This slight reduction in pH increased the undissociated lactic acid fraction from 0.0065 and 0.0068 in the 0.25% GDL treatments to 0.0085 and 0.0091 in the 0.5% GDL treatments (Table 6-9). Which resulted in a 0.36 and 0.90 mmol/L in the concentrations of undissociated lactic acid for 1.5% and 3.0% SL treatments, respectively. The increase in undissociated lactic acid may or may not have been large enough to increase the inhibition of pathogenic bacteria. Therefore, the theory that GDL reduces pH and increases the proportion of undissociated lactic acid may not fully describe the synergistic mechanism between GDL and SL. A more complete explanation may involve the equilibrium that occurs between undissociated GDL and undissociated lactic acid. The pKa values for GDL and lactic acid are 3.7 and 3.86, respectively. The different pKas of GDL and lactic acid indicate that if they are in solution together, GDL will dissociate to form H^+ and GDL^- ions and dissociated lactic acid (LA^-) will accept H^+ ions to form undissociated lactic acid. Therefore, the concentration of undissociated lactic acid will increase and contribute to lactic acid's antimicrobial activity.

Refrigerated storage

Microbial analysis

Statistical analysis of aerobic and anaerobic mesophile and aerobic psychrotrophic growth during refrigerated storage indicated that growth was not affected ($P > 0.05$) by treatment, week or treatment by week interactions (Table 6-10). All treatments exhibited less than 10^2 CFU/g of aerobic and anaerobic mesophilic and aerobic psychrotrophic growth during storage. Refrigeration at 0 to 3°C appeared to inhibit all microbial growth. Therefore, refrigeration eliminated any treatment effects that may have been observed during storage for 8 weeks.

Table 6-10. Probability values ($P > F$) for sources of variation in microbial growth during refrigerated (0 to 3°C) storage

Source	Aerobic Psycrotrophs	Aerobic Mesophiles	Anaerobic Mesophiles
Replication	0.0099	0.0257	0.0356
Treatment	0.7635	0.5980	0.9216
Week	0.8171	0.7254	0.2227
Treatment*week	0.2903	0.4598	0.2688

Salmonella was not isolated from any product during refrigerated storage. The presence of *Salmonella* in the nonrefrigerated product after 3 days of storage, indicated that *Salmonella* survived the cooking process. The absence of *Salmonella* in the refrigerated product indicates that refrigeration temperatures (0 to 3°C) prevented the resuscitation of injured cells during storage. Refrigeration acted as a barrier against the growth of this pathogen and its potential for foodborne illness.

Chemical analysis

Statistical analysis of the TBA values revealed significant ($P < 0.0001$) treatment and week (storage) effects and a significant ($P < 0.0001$) treatment by week interaction as shown in Table 6-11. The mean TBA values as a function of treatment and week are presented in Fig. 6-5. During the eight weeks of storage, the nitrite control exhibited the lowest TBA values, while the control exhibited the highest TBA values during the first four weeks of storage. The TBA values for most of the treatments were relatively stable during storage, with the exceptions of the control and the 0.25% GDL-3.0% SL treatment. The treatment by week interaction appeared to be the result of an elevated TBA value for the 0.25% GDL-3.0% SL treatment and a depressed TBA value for the control at week 8 of storage.

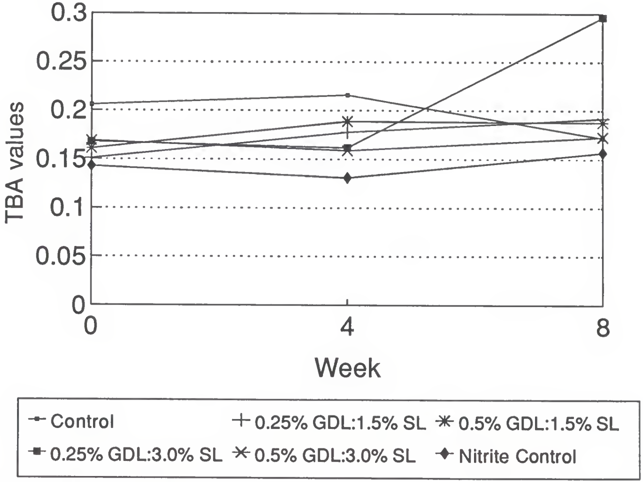

Fig. 6-5—Mean 2-thiobarbituric acid (TBA) values (mg malonaldehyde/kg meat) of restructured pork rolls during refrigerated (0 to 3°C) storage (GDL - glucono-delta-lactone; SL -sodium lactate) (STD ERR ± 0.017, n = 9).

Table 6-11. Probability values (P > F) for sources of variation in a_w, pH and 2-thiobarbituric acid (TBA) values during refrigerated (0 to 3°C) storage

Source	a_w	pH	TBA
Replication	0.0550	0.0001	0.0001
Treatment	0.0001	0.0001	0.0001
Week	0.0001	0.0001	0.0049
Treatment*week	0.3093	0.3707	0.0001

Water activity was affected ($P < 0.0001$) by treatment and week (Table 6-11). Treatment averages are presented in Table 6-12. The control and nitrite control pork rolls exhibited higher ($P < 0.05$) water activities than the pork rolls containing SL and GDL. Suppressed water activity in SL and GDL treated pork rolls was also observed in the nonrefrigerated products. SL was considered to be the cause of reduced water activity because it has been identified as a humectant. The average water activities for each week of analysis are shown in Table 6-13. During refrigerated storage, water activity decreased during the first four weeks and did not significantly ($P > 0.05$) change from weeks 4 to 8. All the water activity values were within the growth range of both spoilage and pathogenic bacteria and the differences were small. However, the slight reduction in a_w caused by GDL and SL may contribute to preservation.

Table 6-12. Mean a_w and pH values of refrigerated, restructured pork rolls by treatment

Treatment	a_w	pH
Control	0.978^b	$6.02^{b,c}$
NO_2 Control	0.978^b	6.04^b
0.25% GDL-1.5% SL[a]	0.971^c	6.01^c
0.5% GDL-1.5% SL	0.972^c	5.87^d
0.25% GDL-3.0% SL	0.969^c	6.01^c
0.5% GDL-3.0% SL	0.967^c	5.88^d

[a] GDL - glucono-delta-lactone, SL - sodium lactate.

[b,c,d,e] Values in same column with different superscripts are significantly different ($P < 0.05$).

Table 6-13. Mean a_w and pH values of refrigerated, restructured pork rolls on a weekly basis

Week	a_w	pH
0	0.975^a	6.02^a
4	0.971^b	5.95^b
8	0.972^b	5.94^b

[a,b] Values in same column with different superscripts are significantly different ($P < 0.05$).

Statistical analysis of pH indicated significant ($P < 0.0001$) treatment and week effects (Table 6-11). Treatment averages are shown in Table 6-12. Pork rolls containing 0.5% GDL were 0.1 pH units more ($P > 0.05$) acidic than the controls and pork rolls containing 0.25% GDL. The average pH for each week of analysis are presented in Table 6-13. During the first four weeks there was a 0.05 unit drop in pH. The pH appeared to stabilize from weeks 4 to 8.

Conclusions

Restructured pork rolls prepared with combinations of 0.25 and 0.5% GDL and 1.5 and 3.0% SL were compared to control pork rolls prepared with and without sodium nitrite. All treatments were stored under refrigerated (0 to 3°C) and nonrefrigerated (18 to 22°C) conditions and evaluated for microbial and physical characteristics during storage. Combinations of SL and GDL were superior to the control and nitrite control at inhibiting microbial growth during nonrefrigerated storage. There were no practical differences in moisture content, protein content, fat content, TBA values, cooking yields or juiciness between the controls and pork rolls containing SL and GDL. Combinations of SL and GDL reduced water activity and increased ash content. The pH was reduced by adding 0.5% GDL. The pork flavor intensity and the saltiness of the pork rolls were enhacnced by the presence of SL and GDL. The presence of bitter and acid off-flavors also tended to increase when SL and GDL concentrations were increased. Overall, SL and GDL enhanced the shelf-life of nonrefrigerated pork rolls without causing extremely detrimental effects on their physical and organoleptic characteristics.

The proposed mechanism of SL and GDL's preservative action is a combination of reduced pH and an equilibrium between undissociated GDL and undissociated lactic acid. Lactic acid has a higher pKa value than GDL which results in increased concentrations of undissociated lactic acid when they are in solution together. The increase in undissociated lactic acid results in increased antimicrobial activity.

CHAPTER 7
SUMMARY AND CONCLUSIONS

This research was performed to investigate the potential use of sodium lactate (SL), glucono-delta-lactone (GDL), Nisaplin (NN), ethylenediaminetetraacetate (EDTA) and their combinations as preservatives for a noncured, nonrefrigerated restructured pork product. The study was performed in a step-wise progression to evaluate the effects of preservative combinations on selected pathogens in a cooked meat media. When various preservatives or preservative combinations were found to be ineffective against selected pathogens, these materials were eliminated from subsequent experiments. When several preservative combinations were observed to be effective against a range of pathogens, the preservative combinations were then evaluated on their ability to preserve restructured pork rolls stored at 18 to 22°C.

Ethylenediaminetetraacetate was removed from the study because it did not inhibit microbial growth at levels currently allowed in cooked sausages. Similarly, NN was removed from the study because it only exhibited antimicrobial activity toward Gram-positive pathogens, and it exhibited antagonistic interactions with GDL and SL. The remaining preservatives, SL and GDL exhibited synergistic effects toward inhibiting all of the pathogens investigated. The broad range of antimicrobial activity exhibited by SL and GDL combinations warranted their utilization in noncured restructured pork rolls.

When subjected to nonrefrigerated (18 to 22°C) storage, the pork rolls containing SL and GDL exhibited less than 10^2 CFU/g of growth after 15 days. Their noncured and cured counterparts without SL or GDL exhibited greater than 10^5 CFU/g of growth after 9 days. During nonrefrigerated storage *Salmonella* was sporadically isolated from all of the pork roll formulations. GDL and SL reduced the frequency at which *Salmonella* was isolated from the nonrefrigerated pork rolls by 19 to 29%, when compared to their noncured and cured counterparts.

This research has shown that SL and GDL are effective for preserving a nonrefrigerated, noncured pork product. Recognizing that restructured pork rolls only represent processed meat products that are coarsely ground and contain few spices or flavoring agents it is important to evaluate their effectiveness in other types of processed meat products stored in nonrefrigerated conditions. Future research with GDL and SL should focus on fresh or cooked sausage products, cooked whole muscle products, fermented dry or semi-dry sausages and GDL-SL interactions with salt and sodium nitrite.

Gould (1996) recognized that the use of bacteriocins effective against the growth of *Clostridium botulinum* would have potential value because they may allow substantial reductions in thermal processing of high water activity-low acid foods, with potential advantages in energy saving and improved product quality. Several research teams (Heil and McCarthy, 1989; Okereke et al., 1990b; McCarthy et al., 1991) have proposed or developed methods to allow milder heat treatment of heat sensitive canned foods. Combinations of GDL and SL have been shown to inhibit the growth of a broad range of foodborne pathogens without thermal processing. The utilization of GDL and SL in combination with mild heat treatments for heat sensitive canned foods should be investigated for their effectiveness against *C. botulinum* and their ability to retain acceptable sensory characteristics.

APPENDIX A
MICROBIAL LEVELS OF MAIN EFFECTS AND TREATMENTS

Table A-1. The average levels ($\log_{10}$ CFU/ml) of *Escherichia coli* O157:H7 in cooked meat media for the main effects of Nisaplin (0, 0.5, 1.0%), disodium ethylenediaminetetraacetate (0, 0.374, 0.745%), sodium lactate (0, 1.5, 3.0%) and glucono-delta-lactone (0, 0.25, 0.5%) after 1 day of incubation at 37°C (STD ERR ± 0.068)

Main Effect	Treatment Concentration (%)		
Nisaplin	0	0.5	1.0
$\log_{10}$ CFU/ml	6.4	6.3	6.0
Ethylenediaminetetraacetate	0	0.374	0.745
$\log_{10}$ CFU/ml	7.6	6.2	5.0
Sodium lactate	0	1.5	3.0
$\log_{10}$ CFU/ml	7.6	6.2	4.9
Glucono-delta-lactone	0	0.25	0.5
$\log_{10}$ CFU/ml	7.0	6.2	5.5

Table A-2. Levels ($\log_{10}$ CFU/ml) of *Escherichia coli* O157:H7 in cooked meat media containing combinations of Nisaplin (0, 0.5, 1.0%), disodium ethylenediaminetetraacetate (0, 0.374, 0.745%), sodium lactate (0, 1.5, 3.0%) and glucono-delta-lactone (0, 0.25, 0.5%) after 1 day of incubation at 37°C (STD ERR ± 0.35)

NN[a] (%)	EDTA[b] (%)	SL[c] (%)	GDL[d] (%)		
			0	0.25	0.5
0	0	0	8.0	8.0	8.0
0	0	1.5	7.9	7.5	7.6
0	0	3.0	7.9	7.6	6.6
0	0.372	0	7.9	7.6	7.6
0	0.372	1.5	7.2	6.9	5.8
0	0.372	3.0	6.8	4.1	3.3
0	0.745	0	7.5	7.4	6.8
0	0.745	1.5	6.4	5.9	3.9
0	0.745	3.0	4.0	3.1	2.3
0.5	0	0	7.9	8.0	7.9
0.5	0	1.5	7.6	7.8	8.0
0.5	0	3.0	7.8	7.3	6.7
0.5	0.372	0	7.8	7.6	7.6
0.5	0.372	1.5	7.1	5.7	5.8
0.5	0.372	3.0	6.6	5.0	3.5
0.5	0.745	0	7.8	7.3	6.6
0.5	0.745	1.5	7.1	4.5	2.8
0.5	0.745	3.0	3.4	2.6	2.1
1.0	0	0	8.0	7.8	8.0
1.0	0	1.5	7.7	7.8	7.4
1.0	0	3.0	7.8	7.3	6.0
1.0	0.372	0	7.5	7.2	7.6
1.0	0.372	1.5	7.4	5.9	4.3
1.0	0.372	3.0	6.1	3.9	2.8
1.0	0.745	0	7.8	6.9	6.8
1.0	0.745	1.5	6.1	4.6	2.3
1.0	0.745	3.0	4.1	2.3	1.8

[a] NN - Nisaplin.

[b] EDTA - disodium ethylenediaminetetraacetate.

[c] SL - sodium lactate.

[d] GDL - glucono-delta-lactone.

Table A-3. The average levels ($\log_{10}$ CFU/ml) of *Escherichia coli* O157:H7 in cooked meat media for the main effects of disodium ethylenediaminetetraacetate (0, 0.0036, 0.0072%), Nisaplin (0, 0.5, 1.0%), sodium lactate (0, 1.5, 3.0%) and glucono-delta-lactone (0, 0.25, 0.5%) after 1 day of incubation at 37°C (STD ERR ± 0.042)

Main Effect	Treatment Concentration (%)		
Ethylenediaminetetraacetate	0	0.0036	0.0072
$\log_{10}$ CFU/ml	7.4	7.4	7.3
Nisaplin	0	0.5	1.0
$\log_{10}$ CFU/ml	7.3	7.5	7.3
Sodium lactate	0	1.5	3.0
$\log_{10}$ CFU/ml	7.9	7.7	6.5
Glucono-delta-lactone	0	0.25	0.5
$\log_{10}$ CFU/ml	7.8	7.5	6.9

Table A-4. Levels ($\log_{10}$ CFU/ml) of *Escherichia coli* O157:H7 in cooked meat media containing combinations of disodium ethylenediaminetetraacetate (0, 0.0036, 0.0072%), Nisaplin (0, 0.5 1.0%), sodium lactate (0, 1.5, 3.0%) and glucono-delta-lactone (0, 0.25, 0.5%) after 1 day of incubation at 37°C (STD ERR ± 0.22)

EDTA[a] (%)	NN[b] (%)	SL[c] (%)	GDL[d] (%)		
			0	0.25	0.5
0	0	0	8.0	7.9	8.0
0	0	1.5	8.0	7.4	7.9
0	0	3.0	7.7	7.1	3.7
0	0.5	0	8.0	7.8	7.9
0	0.5	1.5	7.9	7.9	7.8
0	0.5	3.0	7.8	7.4	5.8
0	1.0	0	8.0	8.0	8.0
0	1.0	1.5	7.9	7.8	7.4
0	1.0	3.0	7.4	7.0	5.1
0.0036	0	0	8.0	7.9	8.0
0.0036	0	1.5	7.9	7.5	7.4
0.0036	0	3.0	7.6	7.1	5.0
0.0036	0.5	0	7.9	7.9	8.0
0.0036	0.5	1.5	7.9	7.8	7.8
0.0036	0.5	3.0	7.6	6.9	5.4
0.0036	1.0	0	7.9	7.8	8.0
0.0036	1.0	1.5	7.8	7.7	7.4
0.0036	1.0	3.0	7.4	6.8	5.4
0.0072	0	0	8.0	7.9	7.7
0.0072	0	1.5	7.6	7.6	7.2
0.0072	0	3.0	7.7	7.0	3.8
0.0072	0.5	0	8.0	8.0	7.9
0.0072	0.5	1.5	7.8	7.6	7.8
0.0072	0.5	3.0	7.7	6.9	4.9
0.0072	1.0	0	8.0	7.9	7.8
0.0072	1.0	1.5	7.9	7.8	7.3
0.0072	1.0	3.0	7.3	6.4	4.7

[a] EDTA - disodium ethylenediaminetetraacetate.
[b] NN - Nisaplin.
[c] SL - sodium lactate.
[d] GDL - glucono-delta-lactone.

Table A-5. The average levels ($\log_{10}$ CFU/ml) of *Salmonella typhimurium*, *Yersinia enterocolitica*, *Listeria monocytogenes* and *Staphyloccocus aureus* in cooked meat media for the main effects of Nisaplin (0, 0.5, 1.0%), sodium lactate (0, 1.5, 3.0%) and glucono-delta-lactone (0, 0.25, 0.5%) after 1 day of incubation at 37°C (STD ERR ± 0.042)

Main Effect	Treatment Concentration (%)		
S. typhimurium			
Nisaplin	0	0.5	1.0
$\log_{10}$ CFU/ml	6.7	7.1	7.0
Sodium lactate	0	1.5	3.0
$\log_{10}$ CFU/ml	7.7	7.0	6.1
Glucono-delta-lactone	0	0.25	0.5
$\log_{10}$ CFU/ml	7.1	7.5	6.2
Y. enterocolitica			
Nisaplin	0	0.5	1.0
$\log_{10}$ CFU/ml	4.1	4.0	3.9
Sodium lactate	0	1.5	3.0
$\log_{10}$ CFU/ml	5.6	3.6	2.7
Glucono-delta-lactone	0	0.25	0.5
$\log_{10}$ CFU/ml	6.5	3.3	2.1
L. monocytogenes			
Nisaplin	0	0.5	1.0
$\log_{10}$ CFU/ml	4.8	1.5	1.5
Sodium lactate	0	1.5	3.0
$\log_{10}$ CFU/ml	3.3	2.5	2.0
Glucono-delta-lactone	0	0.25	0.5
$\log_{10}$ CFU/ml	3.2	2.5	2.1
S. aureus			
Nisaplin	0	0.5	1.0
$\log_{10}$ CFU/ml	5.6	2.3	1.9
Sodium lactate	0	1.5	3.0
$\log_{10}$ CFU/ml	4.5	2.8	2.4
Glucono-delta-lactone	0	0.25	0.5
$\log_{10}$ CFU/ml	3.6	3.3	2.9

Table A-6. Levels ($\log_{10}$ CFU/ml) of *Salmonella typhimurium* in cooked meat media containing combinations of Nisaplin (0, 0.5 1.0%), sodium lactate (0, 1.5, 3.0%) and glucono-delta-lactone (0, 0.25, 0.5%) after 1 day of incubation at 37°C (STD ERR ± 0.37)

NN[b] (%)	SL[c] (%)	GDL[d] (%)		
		0	0.25	0.5
0	0	7.0	7.5	8.0
0	1.5	6.9	7.1	6.1
0	3.0	7.1	7.5	3.4
0.5	0	7.6	8.0	8.0
0.5	1.5	7.4	7.8	7.1
0.5	3.0	6.1	6.9	5.1
1.0	0	7.3	8.0	7.8
1.0	1.5	7.1	7.4	6.6
1.0	3.0	7.5	7.2	4.1

[a] NN - Nisaplin.
[b] SL - sodium lactate.
[c] GDL - glucono-delta-lactone.

Table A-7. Levels ($\log_{10}$ CFU/ml) of *Yersinia enterocolitica* in cooked meat media containing combinations of Nisaplin (0, 0.5 1.0%), sodium lactate (0, 1.5, 3.0%) and glucono-delta-lactone (0, 0.25, 0.5%) after 1 day of incubation at 37°C (STD ERR ± 0.22)

NN[b] (%)	SL[c] (%)	GDL[d] (%)		
		0	0.25	0.5
0	0	7.9	5.9	2.9
0	1.5	7.3	1.9	1.5
0	3.0	5.7	1.9	1.5
0.5	0	7.6	5.9	3.1
0.5	1.5	6.9	2.4	1.7
0.5	3.0	4.8	1.69	1.5
1.0	0	7.8	5.9	3.5
1.0	1.5	6.2	2.4	1.8
1.0	3.0	4.6	1.5	1.5

[a] NN - Nisaplin.
[b] SL - sodium lactate.
[c] GDL - glucono-delta-lactone.

APPENDIX B
SALMONELLA ENRICHMENT METHOD

1. Pre-enrichment
    - a. Aseptically sample 25 g of meat and place in sterile stomacher bag (double bag).
    - b. Add 225 ml of lactose broth.
    - c. Blend for 1 min and 15 s in Stomacher.
    - d. Hold at room temperature for 60 min.
    - e. Test pH - adjust to 6.8 ± 0.2 with 1 N sodium hydroxide or 1 N hydrochloric acid.
    - f. Incubate for 24 ± 2 hr at 35°C.

2. Selective Enrichment
    - a. Gently shake incubated sample mixture.
    - b. Transfer replicate 1 ml portions to 10 ml selenite cystine and tetrathionate broths.
    - c. Incubate for 24 ± 2 hr at 35°C.

3. Isolation of *Salmonella*
    - a. Vortex enrichment culture tubes and streak 3-mm loop of selenite cystine on bismuth sulfite (BS) and xylose lysine desoxylate (XLD).
    - b. Repeat with tetrathionate culture.
    - c. Incubate for 24 ± 2 hr at 35°C. If no growth, incubate another 24 hr.

        On BS - *Salmonella* colonies are black with/without metallic sheen. With time, a brown to black "halo" will develop.

        On XLD - Typical *Salmonella* colonies are pink with/without black centers. Atypical strains will produce yellow colonies with/without black centers.

4. Verification of *Salmonella*
    - a. Inoculate two or more suspect colonies from each selective plating media into triple sugar iron (TSI) and lysine iron (LIA) agars.
    - b. Touch center of suspect colony with sterile inoculation needle and inoculate a TSI tube by streaking the slant and stabbing the butt.
    - c. Without flaming the needle, inoculate an LIA tube by stabbing the butt twice and then streaking the slant.
    - d. Incubate TSI and LIA tubes at 35°C for 24 ± 2 hrs.

e. *Salmonella* in TSI typically produce alkaline (red) slants and acid (yellow) butts, with/without H_2S production (blackening of agar).
f. In LIA typical reaction is an alkaline (purple) butt. Only a distinct yellow butt is a negative reaction.
g. Samples should only be presumed negative if both LIA and TSI are negative.

Equipment and media

Sterile stomacher bags, lactose broth, selenite cystine broth, tetrathionate broth, BS plates, XLD plates, triple sugar iron agar and lysine iron agar.

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BIOGRAPHICAL SKETCH

The author, Lloyd Jason Woolsey, was born on February 29, 1964, to JoAnne Augusta and Durward Willis Woolsey, Jr. He graduated from Southwest Miami Senior High School in June, 1982. In August, he entered the University of Florida, in Gainesville, Florida. During his four years at the University of Florida, he was actively involved in the University's Block and Bridle Club and participated in both the Livestock Judging and Meat Judging Teams. He received a Bachelor of Science in Agriculture from the University of Florida in May, 1986.

After graduation, he was accepted into graduate school at the University of Nebraska in Lincoln, Nebraska, where he received an assistantship from the Animal Science Department. In January, 1987, the author committed his life to Jesus, The Messiah. He is still living that commitment. He received a Master of Science in Agriculture from the University of Nebraska in May, 1989.

In August, 1989, he was accepted into a doctoral program at the University of Florida in Gainesville, Florida, where he received an assistantship from the Animal Science Department. During his doctoral program the author taught the laboratory portion of an undergraduate meat processing class. In his extra time he served as prayer coordinator, executive coordinator and volunteer staff for the University of Florida's chapter of InterVarsity Christian Fellowship.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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